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(57) Abstract

cDNAs coding for an infectious Western Equine Encephalitis virus (WEE) and infectious Venezuelan Equine Encephalitis virus variant IE (VEE IE) are disclosed in addition to cDNA coding for the structural proteins of Venezuelan Equine Encephalitis virus variant IIIA (VEE IIIA). Novel attenuating mutations of WEE and VEE IE and their uses are described. Also disclosed are attenuated chimearic alphaviruses and their uses.

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TITLE OF THE INVENTION

LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

INTRODUCTION

Western equine encephalitis (WEE), eastern equine encephalitis (EEE) and Venezuelan equine encephalitis virus (VEE) are members of the alphavirus genus of the family Togaviridae which is comprised of a large group of mosquito-borne RNA viruses found throughout much of the world. The viruses normally circulate among rodent or avian hosts through the feeding activities of a variety of mosquitoes. Epizootics occur largely as a result of increased mosquito activity after periods of increased rainfall. Western equine encephalitis virus (WEE) was first recognized in 1930 and causes periodic outbreaks of disease in equines. The virus has been detected over much of the western hemisphere from Argentina north to the more temperate regions of central Canada (For a review, see Reisen and Monath [1988] in The Arboviruses: Epidemiology and Ecology, Vol. V. CRC Press, Inc. Boca Raton). Similarly, EEE was first isolated in Virginia and New Jersey in 1933 (Ten Broeck, C. *et al*. [1935] *J. Exp. Med*. **62**:677) and is now known to be focally endemic throughout much of the northern portion of South

America, Central America and the eastern part of Mexico and the United States. Venezuelan equine encephalitis virus has six serological subtypes (I-VI). Two of these subtypes, I and III have multiple variants, two of these variants are of particular interest in this application, variant IE,and variant IIIA also called Mucambo virus. A live, attenuated vaccine (TC-83) for VEE IA/B has been used for immunization equines and at-risk laboratory and field personnel (Birge *et al.* [1961] *Am. J. Hyg.* **73**:209-218; Pittman *et al.* [1996] *Vaccine* **14**:337-343). The vaccine was credited with helping to limit the northward spread of a serious epizootic of VEE originating in South America in the late 1960's. However, the VEE I/AB vaccines have not yet been licensed by the Food and Drug Administration and have been shown to be effective in preventing disease from VEE IA/B infection only. The current VEE vaccines do not adequately protect against the VEE IE variant or the VEE IIIA variant, as disease has occurred in laboratory workers successfully vaccinated with a vaccine derived from VEE IA/B. In addition, recent unprecedented outbreaks of VEE IE in populations of horses in Mexico indicate a need for a VEE IE vaccine. The lack of adequate cross protection with existing IA/B vaccines documents the need for a VEE IE-specific and a VEE IIIA-specific vaccine.

The vaccines currently in veterinary use for WEE, EEE and VEE IA/B throughout the United States and Canada are formalin-inactivated preparations. Inactivated vaccines for EEE and WEE are also available for use by at-risk laboratory personnel. These inactivated vaccines are poorly immunogenic, require multiple inoculations with frequent boosters and generally result in immunity of short duration.

The shortcomings of the available vaccines indicate a need for the development of new vaccines of high immunogenicity which induce a longer lasting immunity for protection against WEE, EEE and VEE subtypes IE and IIIA.

SUMMARY OF THE INVENTION

The present invention satisfies the need mentioned above.

In this application are described live attenuated vaccines for WEE, EEE, VEE IE and VEE IIIA which may provide higher level immunity in humans and equines for many years, and possibly for life. In addition, very large numbers of vaccine doses can be produced from significantly less starting materials than is possible with the existing inactivated products. The vaccine preparations of the present invention comprise full-length cDNA copies of the genomes of WEE or VEE IE which have been altered such that the RNA produced from the cDNA, and the virus produced therefrom is attenuated and useful as a live vaccine for human and veterinary use. The vaccine preparations for VEE IIIA and EEE are novel chimeric viruses which include the newly discovered structural protein genes of VEE IIIA.

The classic methods of deriving live-attenuated vaccines (blind passage in cell cultures) generally result in heterogeneous and undefined products, hence recent attempts to make live vaccines for alphaviruses have relied on genetic engineering procedures.

The alphavirus genome is a single-stranded, positive-stranded RNA approximately 11,400 nucleotides in length. The 5' two-thirds of the genome consist of a non-coding region of approximately 48 nucleotides followed by a single open reading frame of approximately 7,500 nucleotides which encodes the

viral replicase/transcriptase. The 3′ one-third of the genome encodes the viral structural proteins in the order C-E3-E2-6K-E1, each of which are derived by proteolytic cleavage of the product of a single open reading frame of approximately 3700 nucleotides. The sequences encoding the structural proteins are transcribed as a 26S mRNA from an internal promoter on the negative sense complement of the viral genome. The nucleocapsid (C) protein possesses autoproteolytic activity which cleaves the C protein from the precursor protein soon after the ribosome transits the junction between the C and E3 protein coding sequence. Subsequently, the envelope glycoproteins E2 and E1 are derived by proteolytic cleavage in association with intracellular membranes and form heterodimers. E2 initially appears in the infected cell as a precursor, pE2, which consists of E3 and E2. After extensive glycosylation and transit through the endoplasmic reticulum and the golgi apparatus, E3 is cleaved from E2 by furin-like protease activity at a cleavage site having a consensus sequence of RX(K/R)R, with X being one of many amino acids present in the different viruses, and with the cleavage occuring after the last arginine residue. Subsequently, the E2/E1 complex is transported to the cell surface where it is incorporated into virus budding from the plasma membrane (Strauss and Strauss [1994] *Microbiological Rev.* **58**: 491-562). All documents cited herein *supra* and *infra* are hereby incorporated in their entirety by reference thereto.

Because the genome of alphavirus is a positive-stranded RNA, and infectious upon transfection of cells in culture, an "infectious clone" approach to vaccine development is particularly suitable for the alphaviruses. In this approach, a full-length cDNA

clone of the viral genome is constructed downstream from a RNA polymerase promoter, such that RNA which is equivalent to the viral genome can be transcribed from the DNA clone *in vitro*. This allows site-directed mutagenesis procedures to be used to insert specific mutations into the DNA clone, which are then reflected in the virus which is recovered by transfection of the RNA.

Previous work with infectious clones of other alphaviruses has demonstrated that disruption of the furin cleavage site results in a virus which incorporates pE2 into the mature virus. Davis *et al.* (1995, *supra*) found that disruption of the furin cleavage site in an infectious clone of VEE is a lethal mutation. Transfection of BHK cells with RNA transcribed from this mutant clone resulted in the release of non-infectious particles. However, a low level of infectious virus was produced which contained secondary suppressor mutations such that virus containing pE2 was fully replication competent and subsequently shown to be avirulent but capable of eliciting immunity to lethal virus challenge in a variety of animal species.

The genetic basis for attenuation of the VEE TC-83 vaccine and certain laboratory strains of VEE virus have been studied extensively and has led to the development of improved live, attenuated vaccine candidates (Kinney *et al.* 1993, *supra*, Davis *et al.* 1995, *supra*). The approach used in this application is similar to that used for VEE, however, following the VEE example exactly did not result in an adequate vaccine for WEE. Changes in the procedure used for VEE were required, none of which could have been predicted from the VEE work, in order to produce the attenuated live WEE virus of the present invention.

Based upon a comparison of the structural protein gene sequences of WEE and other alphaviruses, the probable furin cleavage site of WEE strain CBA/87 virus is RRPKR. The presence of the extra arginine when compared to the conscensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage at this site might be more complex than that observed with VEE virus. It was necessary therefore to prepare two deletion mutations in the E3-E2 cleavage site of the full-length clone, one which lacks five amino acids and one which lacks four amino acids since it was unknown which mutation, if any, would produce an attenuated virus. The residual arginine in the full-length clone lacking only four amino acids was of concern due to the possibility that other mutations might arise due to the presence of the extra arginine resulting in cleavage by cellular proteases at that site and producing an apparently wild type virus with respect to cleavage of pE2.

Transfection of cultured cells with RNA transcribed from an infectious clone of WEE lacking the furin cleavage site yielded viruses which contained the pE2 of WEE in the mature virus but which were not replication competent. During intracellular replication of the RNA, mutations arise at low frequency, resulting in a small number of replication competent virus. Sequence analysis of these viruses has shown that the lethal effect of the deletion mutations was alleviated by the appearance of second site mutations in the E2 glycoprotein. These viruses are attenuated in mice when administered by subcutaneous or intracranial inoculation. The mice produce high titer neutralizing and ELISA antibody and are protected against a lethal challenge of parental virulent WEE virus.

Therefore, in one aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious western equine encephalitis (WEE) virus RNA transcript. DNA representing the entire genome, not previously available, was prepared by polymerase chain reaction using a series of primer pairs based upon the partial genome sequences previously deposited in Genbank. The 5′ and 3′ ends of the viral genome were unknown and difficult to obtain. The terminal sequence was necessary for efficient replication of the virus since substitution of ends from a similar virus with similar but not identical sequences resulted in an extremely attenuated virus. In order to determine the correct sequence at the 5′ end, a protocol called rapid amplification of cDNA ends (5′-RACE) was used. The full length infectious clone is useful in the production of virulent WEE virus, and introducing and testing attenuating mutations. The production of virulent virus is essential for a formal measure of the degree of attenuation achieved with candidate attenuating mutations and a formal determination of the rate at which reversion to virulence might occur.

In another aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious Venezuelan equine encephalitis virus IE variant (VEE IE) virus RNA transcript (SEQ ID NO: 2). Using oligonucleotides specific to genomic RNA of a VEE IE isolate (GenBank accession no. U34999) (Oberste, *et al.* [1996] *Virology* **219**:314-320), reverse transcriptase polymerase chain reaction was carried out to generate numerous cDNA fragments which were subsequently cloned and used to assemble full-length cDNA of VEE IE. The full length infectious clone is useful, for example, in the production of

virulent VEE IE virus, and introducing and testing attenuating mutations.

In the case of VEE IIIA, the structural protein genes were removed from a full length clone of VEE IA/B and replaced by the VEE IIIA structural genes. The IIIA structural gene sequences were prepared by RT-PCR and include the IIIA 26S promoter and the 3' nontranslated region (3'NTR) flanking the sequences for the structural proteins (SEQ ID NO:3). The VEE IIIA 3' NTR was then replaced with the VEE IA/B NTR, and the modified sequence was cloned back into the VEE IA/B full length clone. The result was a clone in which the nonstructural protein gene sequences were from VEE IA/B and the structural protein gene sequences from VEE IIIA. The virus produced from this chimeric clone replicated efficiently in cell culture, and proved to be completely attenuated in mice. In addition, it was highly immunogenic and protected the vaccinated mice against challenge with virulent, wild-type Mucambo virus (VEE IIIA).

Portions of the cDNA sequences described above are useful as probes to diagnose the presence of virus in samples, and to define naturally occuring variants of the virus. These cDNAs also make available polypeptide sequences of WEE antigens, EEE antigens, VEE IE antigens, and VEE IIIA structural polypeptide antigens encoded within the respective genomes and permits the production of polypeptides which are useful as standards or reagents in diagnostic tests and/or as components of vaccines. Antibodies, both polyclonal and monoclonal, directed against WEE epitopes, EEE epitopes, VEE IE epitopes, or VEE IIIA epitopes contained within these polypeptide sequences are also useful for diagnostic tests, as therapeutic agents, and for screening of antiviral agents.

Accordingly, with respect to polynucleotides, some aspects of the invention are: a purified WEE polynucleotide; a recombinant WEE polynucleotide; a recombinant polynucleotide comprising a sequence derived from a WEE genome or from WEE cDNA; a recombinant polynucleotide encoding an epitope of WEE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transfected with any of these vectors.

Other aspects of the invention are: a purified VEE IE polynucleotide; a recombinant VEE IE polynucleotide; a recombinant polynucleotide comprising a sequence derived from a VEE IE genome or from VEE IE cDNA; a recombinant polynucleotide encoding an epitope of VEE IE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transformed with any of these vectors.

In a further aspect of the invention is a complete sequence of the VEE subtype IIIA structural protein genes useful for diagnostics and vaccine development. Also provided is a chimeric virus containing the structural sequences of VEE IIIA, which is completely attenuated and provides protection against challenge with VEE IIIA virulent virus for use as a vaccine

Another aspect of the invention is a single-stranded DNA sequence comprising a cDNA clone coding for an infectious WEE, the cDNA clone including at least one attenuating mutation therein, the RNA produced from transcription of the cDNA and the virus particles produced from the RNA in a host cell for use as a vaccine.

In another aspect of the invention there is provided a full length WEE cDNA clone containing a

defined deletion mutation useful for attenuating the virus for the identification of suppressor mutations in the virus. The attenuated virus with the cleavage deletion and suppressor mutations are useful as a means to generate an attenuated, live WEE virus vaccine for veterinary and human use.

In a further aspect of the invention is provided a chimeric virus containing nonstructural protein gene sequences from WEE and structural protein gene sequences from any alphaviruses including but not limited to Aura, Barmah Forest, Bebaru, Cabassou, Chikungunya, eastern equine encephalitis, Everglades, Fort Morgan, Getah, Highlands J, Kyzylagach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna, Ross River, Sagiyama, Semliki Forest, SAAR87, Sindbis, Tonate, Una, Venezuelan equine encephalitis, Whataroa, which could be used as a means for attenuating virulent alphaviruses, and vaccine production against other alphaviruses.

Taking advantage of the close evolutionary relationship between WEE and eastern equine encephalitis virus (EEE), a chimeric virus has been constructed in which the structural protein genes of EEE have been inserted into the infectious clone in place of the WEE structural protein genes. The resulting virus is fully replication competent, attenuated, and elicits an immune response in mice which is protective against a lethal challenge with virulent EEE virus.

In addition, depending on the non-WEE sequences substituted for the WEE structural genes, another aspect of the invention includes a means for expressing antigens of other alphaviruses as chimeric alphaviruses for use as potential vaccines for human and veterinary use.

In another aspect of the invention there is provided a full length infectious VEE IE cDNA clone containing a cleavage deletion useful in the identification of suppressor mutations in the virus, the RNA produced from the cDNA and the virus produced from the RNA. The virus with the cleavage deletion and suppressor mutations is useful as a means to generate an attenuated, live VEE IE vaccine virus for veterinary and human use.

In a further aspect of the invention is provided a chimeric virus containing nonstructural sequences from VEE IE and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses.

In addition, depending on the non-VEE IE sequences substituted for the structural sequences of VEE IE, another aspect of the invention includes a means to express antigens of other alphaviruses as chimeric alphaviruses as potential vaccines for human and veterinary use.

In a further aspect of the invention, there is provided a vaccine protective against WEE, the vaccine comprising live attenuated WEE virus in an amount effective to elicit protective antibodies in an animal to WEE and a pharmaceutically acceptable diluent, carrier, or excipient.

In yet a further aspect of the invention, there is provided a vaccine protective against VEE IE, the vaccine comprising live attenuated VEE IE virus in an amount effective to elicit protective antibodies in an animal to VEE IE and a pharmaceutically acceptable diluent, carrier, or excipient.

In another aspect of the invention, there is provided a bivalent vaccine protective against WEE and VEE IE, the vaccine comprising both attenuated WEE and

attenuated VEE IE in an amount effective to elicit protective antibodies in an animal to both WEE and VEE IE and a pharmaceutically acceptable diluent. In addition, it is possible that this vaccine will provide short lived protection against other alphaviruses (Cole and McKinney [1971] *Inf. Immunity* **4**:37-43).

In yet another aspect of the invention, there is provided an inactivated vaccine produced from the live attenuated virus described above. The attenuated virus of the present invention whether whole virus or chimearic virus can be used in producing inactivated virus vaccines. By using an attenuated virus strain, there is a much greater margin of safety in the event that the produce is incompletely inactivated. Starting with an attenuated strain is also much safer during the manufacturing phase, and allows production under lower biocontainment levels.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1A, 1B and 1C. Assembly of the full length cDNA clone of western equine encephalitis virus. Polymerase chain reaction products representing the entire genome of WEE virus were prepared with the primer pairs indicated. Each of the products was cloned into pBluescript KS+. Assembly of the full length clone, pWE2000 in pBluescript was carried out as indicated in the figure and described below. The clones are not drawn to scale. In each of the plasmids, the primers used to generate the PCR

products are indicated as are the restriction endonuclease sites used for the assembly.

Figure 2. Polypeptide profiles of western equine encephalitis viruses. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight marker (MW) are shown in the first lane and molecular weights are indicated in daltons X 10^{-3}. The virus strain is designated above the appropriate lane. Position of contaminating bovine serum albumin is indicated by arrow.

Figure 3. Polypeptide profiles of western equine encephalitis virus (WEE), eastern equine encephalitis (EEE) and chimeric virus, MWE. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight markers (MW) are shown and molecular weights are indicated in daltons X 10^{-3}. Position of contaminating bovine serum albumin is indicated by arrow.

Figure 4. Derivation of virulent VEE IE clone. Constructions of cDNA encoding the entire genome of cloned strain 68U201 are shown. The relevent cloning sites and genetic markers are indicated above each clone. The first full-length clone produced that replicated *in vitro* after transfection of the RNA transcribed from the T7 promoter, was pIE-1006. Further modifications to this clone were carried out in order to obtain pIE-1009 that had the *in vitro* and *in vivo* characteristics of the parental, biological isolate, strain 68U201. With a fully virulent clone, pIE-1009 available, specific attenuating mutations

were introduced into pIE-1009 to generate the attenuated clone, pIE1100. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones.

Figure 5. Construction of IAB-IIIA cDNA chimeric clone pV3A-1000.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to a full length cDNA clone of fully virulent WEE virus specified in SEQ ID NO:1, and a full length cDNA clone of fully virulent VEE IE variant specified in SEQ ID NO:2.

WEE, strain CBA/87, isolated from the brains of an infected horse in Argentina in 1987 (Bianchi *et al.*, [1987] *Am.J. Trop. Med. Hyg.* **49**:322-328) was used as a parent strain in the instant invention. Any other strain which consistently kills 100% of 5 week old C57BL6 mice when inoculated subcutaneously can be used such as B11, for example. The cDNA clone can be generated by any of a variety of standard methods known in the art. Preferably, DNA representing the entire genome can be prepared by polymerase chain reaction using a series of primer pairs based upon the partial genome sequences previously deposited in GenBank. The 5′ terminal sequence of the virus may be determined by 5′-RACE basically as described by Frohman *et al.*([1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**:8998-9002). Assembly of the full length clone can be in a suitable transcription vector such as, for example, pBluescript KS+, using convenient restriction endonuclease sites or the entire genome could be inserted into any plasmid which contains suitable restriction endonuclease cleavage sites for cloning,

an origin of replication so that the plasmid can be propagated in a bacterial host, and a selectable marker gene to maintain the plasmid in the bacterial cell during growth. The DNA sequence preferably has a complementary DNA sequence bonded thereto so that the double-stranded sequence will serve as an active template for RNA polymerase. Hence, the transcription vector preferably comprises a plasmid. When the DNA sequence comprises a plasmid, it is preferred that a unique restriction site be provided 3′ (with respect to the virion RNA sequence) to ("down-stream" from) the cDNA clone. This provides a means for linearizing the DNA sequence to enhance the efficiency of transcription of genome-length RNA *in vitro*.

The complete clone is preferably operatively associated with a promoter region such that the promoter causes the cDNA clone to be transcribed in the presence of an RNA polymerase which binds to the promoter. The promoter is positioned on the 5′ end (with respect to the virion RNA sequence), or upstream from, the cDNA clone. An excessive number of nucleotides between the promoter sequence and the cDNA clone will result in the inoperability of the construct. Hence, the number of nucleotides between the promoter sequence and the cDNA clone is preferably not more than eight, more preferably not more than than 5, still more preferably not more than 3, and most preferably not more than 1. Exemplary promoters useful in the present invention include, but are not limited to, T3 promoters, T7 promoters, and SP6 promoters. It is preferable that the 5′ end of the *in vitro* transcript not have any additional nucleotides preceding the first nucleotide of the viral sequence. At the 3′ end, additional nucleotides can be tolerated in the *in vitro* transcript but are probably lost when

the virus replicates. In most instances, the poly-dA tract at the 3′ end is required for viability of the virus. Selection of the virulent full length clone can be achieved by comparing the LD_{50} of the virus encoded by the cloned cDNA with the LD_{50} of the parent virus used, in the instant example, WEE CBA/87. The ability to produce virulent virus is important; it allows the introduction and testing of attenuation mutations and the attenuated phenotype against a standard: the virulent parent.

Transfection of cells with the RNA transcript coded by the full length genomic cDNA can be achieved by any suitable means, such as, for example, by treating the cells with DEAE dextran, treating the cells with "LIPOFECTIN", and by electroporation. Togavirus-permissive cells, alphavirus-permissive cells, and WEE-permissive and VEE IE-permissive cells are cells which, upon transfection with the viral RNA transcript, are capable of producing viral particles. Togaviruses have a broad host range. Examples of such cells include, but are not limited to, Vero cells, baby hamster kidney cells, chicken embryo fibroblast cells, Chinese hamster ovary cells (CHO), mouse L cells, MRC-5 cells, mosquito cells such as C6-36 cells, to name a few.

In the case of VEE IE, an isolate of VEE IE, strain 68U201, was used. Any isolate known to cause disease in man can be chosen. In addition the ability of a strain to have an easily identifiable phenotype such as, for example, the ability to form large plaques in tissue culture on indicator cell monolayers, is helpful. In the present invention, the full length clone of VEE IE was obtained using oligonucleotide primers specific for the VEE IE strain 68U201 sequence. Reverse transcription-polymerase

chain reaction (RT-PCR) of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned and used to assemble a full-length cDNA in a plasmid such that the cDNA could be precisely transcribed and viral infectious RNA produced. It is also possible to produce the entire genome by polymerase chain reaction by including the promoter sequence in the 5′ end primer thereby producing infectious RNA directly from the PCR fragment. It is also possible to transcribe the viral RNA from a plasmid in the cell by transfection with the appropriate plasmid containing a promoter utilized by cellular RNA polymerases, i.e. the CMV promoter.

To determine virulence of the cloned viral genome, mice can be innoculated subcutaneously with 10^4 plaque forming units of the cloned virus. The clone is considered virulent if all mice die, and not fully virulent if mice do not all die. The LD_{50} of parent VEE IE strain 68U201 is to 1-2 pfu, therefore, if inoculation of 10,000-fold did not cause lethal disease in all mice, it was considered attenuated.

In another embodiment of the present invnetion is provided a cDNA sequence of the entire 26S region of VEE subtype IIIA as well as the structural protein genes specified in SEQ ID NO:3.

DNA or polynucleotide sequences to which the invention also relates include sequences of at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, most preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the WEE, VEE IE, or VEE IIIA nucleotide sequence. Preferably, the sequence of the region from which the polynucleotide is derived is homologous to or complementary to a sequence which is

unique to the virus. Whether or not a sequence is unique to the virus can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks, e.g., GenBank. Regions from which typical DNA sequences may be derived include but are not limited to, for example, regions encoding specific epitopes, as well as non-translated regions.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence of the alphaviruses, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction assays and for the discovery of other alphavirus sequences.

A polypeptide or amino acid sequence derived from the amino acid sequence of alphavirus, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with as a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid

sequence, it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system.

Once a complete viral genomic cDNA is cloned, attenuation of the virus is possible. An attenuating mutation refers to a nucleotide mutation or amino acid coded for in view of such a mutation which results in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with standard terminology in the art. The attenuating mutation may be a substitution mutation or an in-frame deletion mutation.

Novel WEE attenuating mutations disclosed herein which may be used to carry out the present invention include deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for glutamic acid at codon 182 at E2, or deletion of five amino acids at the furin cleavage site in combination with a subsitution of lysine for glutamic acid at codon 181 at E2. Additionally, certain mutations placed in the non-coding region at the 5′ end of the genome have been found to be attenuating, specifically, a C to T change at nucleotide 7, an A to G change at nucleotide 13, a T to A change at nucleotide 25 and deletion of an A at nucleotide 22. These novel attenuating mutations may be inserted together in a cDNA clone encoding WEE virus resulting in an attenuated WEE which is reflected by 100% survival of mice inoculated by subcutaneous and intracranial routes. Such an attenuated live virus is immunogenic and protective against a lethal virus challenge.

Novel attenuating mutations can be discovered in the VEE IE by introducing mutations which are not reparable by the viral RNA replication process. A

preferable mutation is the deletion of the four amino acids of the furin-like cleavage site between the E3 and E2 proteins. Transfection of the mutant viral genome into cells can result in the suppression of the lethal effect of the deletion mutation due to the error prone process of alphavirus replication. Once efficiently replicating viral progeny is generated they can be detected by plaque assays and analyzed for the presence of pE2 protein which indicates that the virus contains the deletion mutation. Attenuated but yet immunogenic virus with a cleavage deletion mutation and suppressor mutation(s) could be tested for its ability to protect animals from challenge with virulent VEE IE.

Attenuating mutations may be introduced into cDNAs encoding live WEE or VEE IE by any suitable means, such as site-directed mutagenesis (Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) or Current Protocols in Molecular Biology, Ausubel, F. M *et al.* (Eds.) John Wiley & Sons, Inc., for general cloning methods.).

In another embodiment of the present invention is provided a chimeric virus containing nonstructural sequences from one alphavirus and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses. By "Structural sequences" as used herein is meant sequences encoding proteins which are required for encapsidation (e.g., packaging) of the viral genome, and include the capsid protein, E1 glycoprotein, and E2 glycoprotein. By "nonstructural sequences" is meant nonstructural protein sequences, or sequences which encode viral RNA

polymerase(s) proteins. Viruses from which structural sequences can be used in the chimeric virus using WEE "nonstructural genes" as the backbone clone can include for example, all strains of WEE, EEE, and Sindbis, Aura, Barmah Forest, Bebaru, Bijou Bridge, Cabassou, Chikungunya, Everglades, Fort Morgan, Getah, Highlands J, Kyzylagach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna, Ross River, Sagiyama, Semliki Forest, SAAR87, Tonate, Una, Venezuelan Equine Encephalitis, Whataroa, to name a few. Acceptable structural protein genes would include a nucleocapsid protein capable of both packaging the chimeric viral genome and which can interact with the glycoproteins to initiate particle assembly. Chimearic virus is constructed by excision of the structural protein genes of the backbone virus and replacement with the desired structural protein genes from another virus. This can be accomplished in one of several ways. For example, site-directed mutagenesis can be used to excise the structural protein genes and leave a restriction endonuclease digestion site at the point of deletion. The structural protein genes of another alphavirus would then be cloned into that restriction site. Any virus obtained after transfection of cells with RNA transcribed from that clone would by definition be a chimeric virus.

In the case where the first and second viruses are closely related, another method can be used wherein cloned structural cDNA sequences of a second alphavirus can be digested at restriction enzyme sites which both viruses have in common. The cDNA fragments of the second virus can then be cloned into the homologous sites in the first virus structural protein locus such that the resulting structural protein genes of the chimeric are a composite of both. Other

methods for producing a chimearic virus are known to people in the art (Kuhn *et al.* [1996] *J. Virology* **70**:7900-7909).

In another embodiment, the attenuated viruses of the present of invention can be used to prepare replicon expression systems. A replicon expression system consists of three components. The first is a replicon which is equivalent to a full length infectious clone from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in *trans*. This is typically done with two helpers. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequence for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the

replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the heterologous gene cloned in place of the structural proteins. The heterologous gene product is then detected by the host immune system and appropriate immune response is then mounted.

The WEE and VEE IE replicons can be used to express heterologous genes of interest as well as a means for expressing antigens or immunogenic proteins and peptides of interest, *in vitro* or *in vivo*. The immunogenic protein or peptide, or "immunogen" may be any immunogen suitable for inducing an immune response protective against a pathogen from which the immunogen is derived, including but not limited to microbial, bacterial, protozoal, parasitic, and viral pathogens. For example, the immunogen can be the expression product of any heterologous gene of interest, including influenza hemagglutinin, lassa fever nucleocapsid and glycoproteins, portions of bacterial toxin genes, HIV glycoprotein, Ebola glycoprotein, to name a few.

In yet another embodiment, the present invention provides inactivated virus vaccines produced from live attenuated virus preparations, either as virus with attenuating mutations as has been described for WEE and VEE IE or chimeric viruses described above for EEE and VEE IIIA. The inactivation of live virus is well

known in the art and can be performed, for example, by the use of formalin. Inactivated attenuated virus vaccine has a greater safety margin both as a final vaccine in case of incomplete inactivation, and during the manufacturing process allowing production under lower biocontainment levels.

Subjects which may be administered the live attenuated or inactivated attenuated viruses and vaccine formulations disclosed herein include both humans and animals (e.g. horse, donkey, pigs, mice, hamster, monkey, birds).

Vaccine formulations of the present invention comprise an immunogenic amount of a live attenuated virus, or a combination of live attenuated viruses as a multivalent vaccine, as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the attenuated virus sufficient to evoke an immune response, particularly an immune response to the protein or peptide encoded by the heterologous RNA carried by the virus, in the subject to which the virus is administered. An amount of from about 10^1 to 10^5 plaque forming units of the live virus per dose is suitable, depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the live attenuated viruses disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface.

Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the virus as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

In another embodiment, the present invention relates to antibodies specific for the above-described virus. For instance, an antibody can be raised against any of the viral proteins or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to a polypeptide of the present invention. Material and methods for producing antibodies are well known in the art (see for example Goding, in, <u>Monoclonal Antibodies: Principles and Practice</u>, Chapter 4, 1986). The antibodies can be used to monitor the presence or activity of alphaviruses and potentially as a therapeutic agent.

In a further embodiment, the present invention relates to a method of detecting the presence of WEE, EEE, VEE IIIA or VEE IE viral infection or antibodies against these viruses, if present, in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a

microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of WEE, EEE, VEE IIIA or VEE IE virus described above, and contacting it with the serum of a person suspected of having a viral infection. The presence of a resulting complex formed between the virus and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as colorimetry or microscopy. This method of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infections.

In yet another embodiment, the present invention relates to a method of detecting the presence of WEE, EEE, VEE IIIA or VEE IE viruses in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), antibodies specific for WEE and/or VEE IE, and contacting it with serum or tissue sample of a person suspected of having a WEE or VEE IE viral infection. The presence of a resulting complex formed between virus in the serum and antibodies specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infection.

In another embodiment, the present invention relates to a diagnostic kit which contains WEE, EEE, VEE IE, or VEE IIIA virus and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to WEE and VEE IE in serum or a tissue sample. Tissue samples

contemplated can be obtained from birds, monkey, human, or other mammals.

In yet a further embodiment, the present invention relates to DNA or nucleotide sequences for use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA virus using the reverse transcription-polymerase chain reaction (RT-PCR). The DNA sequence of the present invention can be used to design primers which specifically bind to the viral RNA for the purpose of detecting the presence, absence, or quantitating the amount of virus. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides. Reagents and controls necessary for PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of viral sequences, for example by gel fractionation, with or without hyridization, by radiochemistry, and immunochemical techniques.

In yet another embodiment, the present invention relates to a diagnostic kit which contains PCR primers specific for WEE, EEE, VEE IE or VEE IIIA and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA in a sample using PCR. Samples contemplated can be obtained from birds, human, or other mammals.

In another embodiment, the present invention relates to a method of reducing WEE, EEE, VEE IE, or VEE IIIA viral infection symptoms in a patient by administering to said patient an effective amount of anti WEE, anti EEE, anti VEE IE, or anti VEE IIIA antibodies, or protective serum from an immunized animal. When providing a patient with antibodies, the dosage of administered agent will vary depending upon

such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from about 1pg/kg to 10 mg/kg body weight of patient, although a lower or higher dosage may be administered.

In another embodiment, the present invention relates to a method for overcoming vaccine interference in alphavirus-immune individuals. Alphavirus interference has been documented in animals and people since the 1960's. This phenomenon occurs when a live-attenuated vaccine is administered to animals or people with existing immunity to heterologous alphaviruses. Pre-existing immunity may be acquired by vaccination or infection. This presents a significant limitation to the usefulness of the current live-attenuated alphavirus vaccines, especially since the cross-reactive immunity does not protect adequately against challenge with virulent heterologous alphaviruses. Formalin-inactivated vaccines are not an acceptable alternative as they have significant limitations with regard to the quality and duration of protective immunity and require multiple inoculations and periodic boosters. The attenuated WEE, EEE, VEE IIIA and VEE IE virus vaccines of the present invention contain mutations in the viral glycoprotein sequences that may alter the sequence, conformation, and/or accessibility of cross-reactive epitopes. Alterations in epitopes that prevent binding by cross-reactive antibodies may also bypass interference in alphavirus-immune individuals. Eliminating the problem of interference would permit the WEE and VEE IE attenuated virus vaccines to be used in alphavirus-immune animals or people to induce

protective immunity to western equine encephalitis virus and/or to Venezuelan equine encephalitis virus variant IE. Long-lasting protective immunity to both parenteral and aerosol challenge would be expected after vaccination with the live-attenuated vaccines of the present invention, and provide an additional advantage over the use of inactivated vaccines which induce short-lived responses that do not protect against mucosal challenge.

Having now described the invention, the following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

Attenuated WEE

The following materials and methods were used in the examples that follow.

Viruses and cells. Western equine encephalitis virus, strain CBA/87 (Bianchi *et al.* 1988) and eastern equine encephalitis virus, strain Fla91-4679 (Mitchell *et al.*[1992] *Science* **257**:526-7) were grown in BHK cells in EMEM containing 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. Primary chicken embryo fibroblasts were grown in EMEM containing 5% fetal bovine serum. Baby hamster kidney cells and Vero cells were grown in EMEM containing 10% fetal bovine serum.

cDNA cloning. Genomic RNA was prepared from purified virus by phenol:chloroform extraction and ethanol precipitation. Initially, cDNA was prepared by the method of Gubler and Hoffman ([1983] *Proc.*

Natl. Acad. Sci. U.S.A. **85**:5997-6001). Subsequently, DNA representing the entire genome was prepared by polymerase chain reaction using a series of primer pairs (Table 1, Figure 1) based upon the partial genome sequences previously deposited in GenBank. Each PCR product was cloned into pCRII (Invitrogen). The 5′ terminal sequence of CBA/87 virus was determined by 5′-RACE basically as described by Frohman *et al.* ([1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**:8998-9002). The oligonucleotide, CBA/87 5′, consisted of an *Sst*II site, the promoter for bacteriophage T7 RNA polymerase followed by one G and 14 nucleotides of the 5′ terminus of CBA/87 terminal sequence was paired with ns1962 and used to amplify the terminal 1.9 kb of the WEE genome. Clone pWE2000 representing the entire genome was assembled in pBluescript KS+ through the use of convenient restriction endonuclease sites.

Table 1. Oligonucleotide Primers for Preparation of WEE PCR Products

CBA/87 -T7-Sst2	GTCACCGCGGTAATACGACTCACTATAGATAGGGCATGGTATAGAG (SEQ ID NO:4)
NS1962	TCACCTTATTCTGGAACACATCAG (SEQ ID NO:5)
WEE-7	TCGGAGGAAGGCTGATGAAAC (SEQ ID NO:6)
WEE-10	TCGGATCCGATGAGAAAATATACGCTCCC (SEQ ID NO:7)
WEE-17	GACTGGATCCGCAAACCAGTCCTGTTCTCAGG (SEQ ID NO:8)
WEE-18	GCATGGATCCAGCATGATCGGAAATGTCTTGTC (SEQ ID NO: 9)
WEE-5	TCGGATCCACCGCCAAAATGTTTCCATAC (SEQ ID NO:10)
WEE-3	TCGGGATCCCCGGAACATTTGGC (SEQ ID NO:11)
WEE-2	CTGCTTTTCATGCTGCATGCC (SEQ ID NO:12)
WEE-Not	CGATGCGGCCGCTTTTTTTTTTTTTTTTGAAATTTTAAAAAC (SEQ ID NO:13)
WEE-CL2	CAGCGTGAAGTCATCGGTAATGCTGCGTGATGGACATTTCAAG (SEQ ID NO:14)
WEE-CL1	CAGCGTGAAGTCATCGGTAATGCTTGATGGACATTTCAAG (SEQ ID NO:15)

For ease of subsequent site-directed mutagenesis of the structural protein genes, two cassettes representing the 5′ terminal 7.6 kb, plasmid pWE5′-18, and 3′ 4.2 kb, plasmid pWE3′-17, of the genome were prepared. Full length clones were assembled by digestion of the pWE5′-18 with *Bln*I and *Not*I and insertion of a 4.1 kb *Bln*I-*Not*I fragment prepared from the plasmid pWE3′-17 or its mutagenized derivatives.

A cassette containing the structural genes of eastern equine encephalitis virus strain Fla91-4679 was prepared by RT-PCR. The cassette was digested with *Bln*I and *Not*I and the 4.0 kb fragment was ligated to pWE5′-18 which had been similarly digested. The resulting plasmid was designated pMWE-7.

Mutagenesis of the furin cleavage site. Two oligonucleotides, WEE-CL2 and WEE-CL which bracket the presumed furin cleavage site, RRPKR, between the E3 and E2 glycporoteins were used to delete the 5 and 4 codons, respectively. Plasmid pWE3′-17 was used as template for mutagenesis. WEECL2 and WEECL were paired with primer WEE-5 to generate PCR products of 1.4 kb. The PCR products were purified and paired with WEE-3 for 10 cycles of PCR utilizing WE3′-17 as template. Additional WEE-5 primer was added to 500 nM and PCR was carried out for an additional 20 cycles. The 2.3 kb products were purified, digested with *Bst*EII and *Nco*I and ligated into plasmid pWE3′-17 which had been digested with *Bst*EII and *Nco*I. Clones containing the mutation were identified by loss of the *Ngo*M1 site in the sequences encoding the furin cleavage site. The sequences of the mutations were confirmed by sequencing and the mutagenized pWE3′-17 cassettes were ligated to pWE5′-18 as described above to yield full length clones pWE2200 and pWE2100, respectively.

Identification of secondary mutations in virus derived from pWE2100 and pWE2200. Virus released from cells electroporated with RNA transcribed from pWE2200 were plaque-purified and grown into stock preparations. Supernatant from cells transfected with RNA transcribed from pWE2100 was diluted 10-fold and inoculated onto BHK cells. The supernatant was collected 48 hours later and the RNA was extracted directly from the culture fluid. For each mutant virus and the parent CBA/87 virus, viral RNA was extracted with Trizol LS (Life Technologies, Inc., Gaithersburg, MD) and the glycoprotein genes of each were amplified by reverse transcription-polymerase chain reaction amplification. The PCR products were purified (PCR Prep, Promega Inc., Madison, WI) and sequenced on an ABI 373 sequencer using fluorescent-tagged terminators.

Transcription and transfection. Purified plasmid was digested with *Not*I, phenol extracted and ethanol precipitated. Typically 0.5-1 ug of linearized DNA was transcribed *in vitro* by T7 RNA polymerase (Ribomax, Promega, Madison, WI) in the presence of 3 mM m7GpppGp (Pharmacia, Piscataway, NJ). Electroporation of BHK or CEF cells with 0.4 ug of RNA was done as described (Liljestrom *et al.* [1991] *Bio/Technology* **9**:1356-1361). The cells were then seeded into T-75 flasks with 20 ml of medium and observed for cytopathology at 24 and 36 hours after electroporation. Virus was harvested when the cells displayed significant cytopathology and approximately 50% were detached from the plastic. Virus titers were determined by plaque assay on Vero and BHK cells.

EXAMPLE 1

Preparation of an infectious clone of WEE strain CBA/87.

One goal of this study was to prepare a full length cDNA clone of fully virulent WEE virus such that mutations leading to an attenuated phenotype could be identified. WEE, strain CBA/87, isolated from the brain of an infected horse in Argentina in 1987 (Bianchi *et al.* [1988] *Am. J. Trop. Med. Hyg.* **49**:322-328), was chosen as the parent virus as it consistently kills 100% of 5 week old C57BL6 mice when inoculated subcutaneously, allowing development of a convenient animal model to assess the relative effects on virulence of the attenuating mutations. Portions of the sequence of several strains of WEE virus had been determined previously and were used as a basis for primers to prepare amplification products representing the entire genome of the CBA/87 which were cloned in pCRII. Full length clones were assembled in pBluescript KS+ using convenient restriction sites as shown in Figure 1. The first infectious clone pWE1000, contained the 5' terminal 20 nucleotides from eastern equine encephalitis virus. This clone produced viable virus which was highly attenuated exhibiting an subcutaneous LD_{50} in mice of approximately 1.2 X 10^6 PFU compared to the CBA/87 parent virus where LD_{50} was approximately 22.

A second clone with an authentic WEE 5'-terminus, pWE2000, was used for all subsequent experiments. Transfection of CEF or BHK cells with RNA transcribed from pWE2000 resulted in complete destruction of the monolayers within 36 hours and titers $>10^8$ PFU/ml were obtained by infection of either cell type with the resulting virus. Subcutaneous inoculation of 5 week

old C57/B16 mice with WE2000 results in death within 9 days and the LD_{50} of 75 PFU is only slightly higher than the LD_{50} of 22 PFU of CBA/87 parent virus, Table 2. It should be noted that animals surviving the lower doses of virus challenge gave no serological evidence of infection. This level of virulence for WE2000 virus was considered sufficient to allow for further characterization of the mutations necessary for attenuation of the virus.

Table 2. C57 Black/6 Mice Inoculated Subcutaneously with CBA/87 Parental virus and WE2000 Recombinant Virus

Virus Strain Dose	Mortality	Mean Day to Death (Days)	Prechallenge ELISA	Challenge (S/T)
CBA/87				
$3X10^5$	10/10	8.4	-	-
$3X10^4$	10/10	8.9	-	-
$3X10^3$	10/10	9.2	-	-
$3X10^2$	7/10	9.9	<100	0/3
$3X10^1$	3/10	10	<100	0/7
3	0/10	--	<100	0/10
WE2000				
$1X10^7$	10/10	8.7	-	-
$1X10^6$	10/10	9.0	-	-
$1X10^5$	10/10	9.2	-	-
$1X10^4$	10/10	9.3	-	-
$1X10^3$	9/10	10.0	<100	0/1
$1X10^2$	3/10	10.0	<100	0/7
$1X10^1$	0/10	-	<100	0/10
1	0/10	-	<100	0/10

EXAMPLE 2

Preparation of cleavage mutants of CBA/87.

Davis *et al.*([1995] *Virology* **212**:102-110) have demonstrated that deletion of the furin cleavage site between E3 and E2 glycoproteins of VEE virus is a lethal mutation. However, prolonged incubation of cells which had been transfected with RNA derived from

full length clones with the deletion, resulted in the eventual appearance of virus which was replication-competent and attenuated in mice (Davis *et al.*, 1995, *supra*). Based upon a comparison of the predicted structural protein sequences of WEE and VEE, the probable cleavage site of CBA/87 virus is RRPKR. The presence of the extra arginine when compared to the conscensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage site of WEE virus might be more complex than that observed with VEE virus. We therefore, prepared two different deletion mutations in the E3-E2 cleavage site of the pWE2000 clone, pWE2100 which lacks five amino acids, RRPKR, and pWE2200 which lacks four amino acids, RPKR.

Development of CPE in cells after electroporation of RNA transcribed from pWE2100 and pWE2200 was delayed for 48 to 72 hours compared with pWE2000. In two instances, pWE2100 did not induce significant CPE in the transfected cells despite the fact that approximately 10^6 PFU/ml were released into the medium of transfected cells. When assayed by plaque formation on Vero cells, both supernatants yielded extremely small plaques after 72 hours which never increased beyond 2 mm in diameter. In contrast, pWE2000 virus yields large plaques after 48 hours which enlarge to approximately 1 cm after 5 days under a 0.5% agarose overlay. The small plaque phenotype of the mutant viruses is stable after 3 passages in Vero or BHK cells, which is the limit to the passage of the virus used in these experiments.

Analysis of the structural proteins of the WE2100 and WE2200 viruses by SDS-PAGE (Figure 2) shows that in each instance, the deletions at the cleavage site result in a virus which lacks E2 protein and contains a larger protein, presumably pE2, indicating that

deletion of the presumed cleavage site eliminated cleavage at this site.

The lack of rapid cytopathology after transfection of BHK cells with transcripts of pWE2200 and pWE2100 suggested that the mutants were non-viable and that the infectious virus subsequently detected by plaque assay was due to secondary mutations arising during the replication of the RNA as reported previously for deletion mutants of VEE (Davis *et al.*, 1995, *supra*).

Three plaque isolates from WE2200 virus were chosen for further characterization. All isolates grew to high titer and exhibited a small plaque phenotype. The isolates were sequenced over the entire glycoprotein reading frame. As shown in Table 3, isolates 2215 and 2220 have a mutation of Glu to Lys at position 181 of the E2 glycoprotein. Strain WE2219 carries a single Glu to Lys at position 182 of the E2 protein. Strain WE2215 also has a conservative Val to Ala change at position 211 of the E2 glycoprotein. WE2220 has a Glu to Gly change at position 2 of the E1 glycoprotein and a Phe to Ser change at position 382 of the E1 glycoprotein.

Table 3. Genotypes of Recombinant WEE Virus Strains

Virus[1]	Cleavage Site	E2[2]	E1[2]
CBA/87	RRPKR	P(102),E(181),E(182)	E(2),F(257),P(382)
vWE2100	------	K(182)	
pWE2102	------	K(182)	
vWE2215	R-----	K(181), A(211)	
vWE2219	R-----	K(182)	
vWE2220	R-----	K(181)	G(2), S(382)

1. p indicates viruses prepared by mutagenesis of infectious clones.
2. Amino acid at position indicated in parenthesis.

In order to determine a consensus of the mutations appearing in virus produced from the pWE2100 RNA, the sequences of the glycoprotein genes were determined directly from cDNA prepared by RT-PCR of the genomic RNA extracted from virus released from BHK cells infected with virus released from the transfected cells. Sequence analysis of glycoprotein genes of WE2100 virus from the transfection supernatant revealed only two mutations. As seen previously in WE2219, WE2100 also had a Glu to Lys change at position 182 of the E2 glycoprotein (Table 3).

In order to determine which of the mutations identified in virus released from cells transfected with RNA transcripts from pWE2200 and pWE2100 served as the suppressor of the lethal effect of the cleavage deletion mutation, the mutations were individually placed into the pWE2200 and pWE2100 clones by site-directed mutagenesis. As shown in Table 4, three subclones of WE2200 were produced, and based upon the ability to induce CPE in BHK cells, it was demonstrated that the Glu to Lys change at position 181 of the E2 glycoprotein was necessary and sufficient to restore the ability of the WE2200 clone to encode replication competent virus. Similarly, placement of the Glu to Lys change at position 182 of the E2 glycoprotein was also sufficient to restore the ability of the WE2100 clone to encode replication competent virus. When the Glu to Lys changes at E2 position 181 or 182 were inserted into the parental infectious clone pWE2000, the resulting virus

exhibited a small plaque phenotype on Vero cells as noted for each of the cleavage deletion mutants.

Table 4. Effect of site-directed mutagenesis on restoration of cytopathogenicity of pWE2200 and pWE2100

Strain	Cleavage site	E2	E1	Viability
pWE2000	RRPKR			Yes
pWE2200	R----			No
pWE2221	R----		G(2)	No
pWE2222	R----	K(181)	G(2)	Yes
pWE2223	R----	K(181)		Yes
pWE2100	-----			No
pWE2102	-----	K(182)		Yes

Although the cleavage deletion mutations in pWE2200 and pWE2100 differed by a single amino acid, the results indicate that the mutations at E2 residues 181 and 182 are both capable of restoring viability of the virus and appear to be equivalent.

EXAMPLE 3

Attenuation of WE2000 virus.

C57/BL6 mice are uniformly susceptible to lethal challenge by western equine encephalitis virus until approximately 9 weeks of age. Subcutaneous inoculation of five- or eight-week-old C57/BL6 female mice with CBA/87 or WE2000 viruses routinely results in lethal encephalitis after 8-9 days (Table 2). As noted previously, the WE2000 is slightly less virulent than the CBA/87 parent. The virulence of the progeny virus derived from pWE2100 and pWE2200 infectious clones were determined by subcutaneous inoculation of C57BL6 mice. In each instance, the viruses were

significantly attenuated compared to virus produced from parent WE2000 clone. However, infection of mice with increasing doses of WE2215, WE2219 or WE2220, resulted in sporadic deaths with slightly extended periods prior to death compared to the parental virus (Table 5). These results indicated that deletion of only four amino acids from the cleavage site was inadequately attenuating, unlike the results obtained with VEE virus by Davis *et al.* (1995) and the viruses derived from WE2200 were not characterized further.

Table 5. C57BL6 Mice Inoculated Subcutaneously with WE2200 Cleavage Deletion Mutants

Virus Strain Dose	Mor-taliy	Mean day to Death	Prechallenge ELISA	Prechallenge Neut	Chal-lenge	Post Challenge ELISA	Post Challenge Neut
CBA/87							
10^3	10/10	9.1					
10^5	10/10	7.7					
WE2000							
10^3	10/10	9.2					
10^5	10/10	9.1					
WE2215							
10^3	1/10	12	504 (3/9) 100 (6/9)	<20(9/9)	8/9	8300	761
10^5	1/10	14	253 (7/9) 100 (2/2)	<20(9/9)	9/9	12800	403
WE2219							
10^3	0/10	-	283(2/8) 100 (6/8)	<20(10/10)	6/10	14368	2281
10^5	2/10	12	200 (4/8) 100 (4/8)	<20(8/8)	8/8	11738	1522
WE2220							
10^3	1/10	11	606 (5/9) 100 (4/9)	20(3/9) <20 (6/9)	5/9	14703	1280
10^5	2/10	14	800 (7/8) 100 (1/8)	20 (2/8) <20 (6/8)	8/8	9051	761

When C57BL6 mice were inoculated subcutaneously with the uncloned WE2100 progeny virus, there were no deaths at any dilution, even at doses of 10^7 PFU per mouse (Table 6). However, some of those mice inoculated subcutaneously with 10^5 PFU or less remained susceptible to a lethal challenge with the virulent CBA/87 virus.

Table 6. C57 Black/6 Mice Inoculated Subcutaneously with WEE Virus Strain WE2100

Virus strain Dose	Mortality[1] (%)		Mean Day to Death	Challenge[2] (%)
WE2100				
10^3	0/10	(0)	-	4/10 (40)
10^4	0/10	(0)	-	7/10 (70)
10^5	0/10	(0)	-	8/10 (80)
10^6	0/10	(0)	-	10/10 (100)
10^7	0/10	(0)	-	10/10 (100)

1. Expressed as animals dying/animals tested
2. Expressed as animals surviving/animals tested

WE2102 virus was demonstrated to be highly attenuated and killed only two of twenty mice when inoculated subcutaneously with the highest dosage of virus (10^7 PFU). All mice were challenged 3 weeks later with 10^5 PFU of CBA/87 virus. Mice previously immunized with 10^5 PFU or more of WE2102 survived with no noticeable symptoms. Thus, an effective immunizing dose of WE2102 is at least 100 fold less than that required to kill C57BL6 mice. These results further indicate that the Glu to Lys change at position 182 of the E2 glycoprotein is responsible for restoring viability to viruses containing a deletion of the furin cleavage site in the WEE glycoproteins and that WE2102 virus is an effective attenuated vaccine virus.

Table 7. Subcutaneous Inoculation of C57BL/6 Mice with recombinant WEE virus strain 2102 confers protection against a lethal challenge

Virus Strain Dose	Mortality[1] (%)	Mean Day to Death	Challenge[2] (%)
WE2100			
10^3	0/10 (0)		3/10 (30)
10^5	1/10 (10)	16	9/9 (100)
10^7	0/10 (0)		10/10 (100)
WE2102			
10^3	0/20 (0)	-	9/20 (45)
10^5	0/20 (0)	-	18/20 (90)
10^7	2/20 (0)	14	18/18 (100)
WE2000			
10^5	10/10 (100)	9.2	

1. Expressed as animals dying/animals tested
2. Expressed as animals surviving/animals tested

Attenuated VEE IE

EXAMPLE 4

Sequence Analysis. Genomic RNA from VEE IE Strain 68U201 was isolated and reverse transcription of the genomic RNA followed by polymerase chain reaction (RT-PCR) was used to generate cDNA of the virus. Initial sequencing of the strain 68U201 genome employed oligonucleotide primers based on existing VEE IE sequence and VEE IA/B sequence. After a partial sequence of strain 68U201 was determined, oligonucleotides specific to the strain 68U201 sequence were used to obtain a complete sequence of the strain 68U201 viral genome. The exact 5′ end of the genome was determined by PCR/RACE technique (Frohman *et al.* [1988] *Proc. Natl. Acad. Sci. U.S.A.* 85:8998-9002). The entire strain 68U201 viral genomes consists of 11,464

nucleotides, excluding the poly-A sequence (Oberste *et al.* [1996] *Virology* **219**: 314-320).

EXAMPLE 5

Construction of Full-length, Live Clones

Using oligonucleotides specific to the VEE IE strain 68U201 sequence, RT-PCR of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned. These clones were used to assemble a full-length cDNA of strain 68U201 in a plasmid situated such that the cDNA could be precisely transcribed in an *in vitro* transcription reaction employing T7 polymerase. The first nucleotide downstream of the T7 promoter is a G followed by a cDNA encoding the entire strain 68U201 genome, including poly-A sequence. For the purposes of run off *in vitro* transcription, a unique endonuclease restriction site (*Not*I) follows the poly-A sequence (Figure 4). DNA sequences encoding the T7 promoter and the strain 68U201 genome were cloned into a suitable plasmid for propagation and selection in *E. coli*. Oligonucleotides relevant to construction of full-length infectious clones are shown in Table 8.

Table 8

0077	CTAAGAGGGGCCCCTATATC (SEQ ID NO:16)
0111	GCGGAATTCTAATACGACTCACTATAGATGGGCGGCGCATGAGAG (SEQ ID NO:17)
0113	TGACCGCGGGACCTCTGTCCAC (SEQ ID NO:18)
0126	AAGTGCATCGATTCAGCG (SEQ ID NO:19)
0136	CTGAAATGTCCAGGATCCACGGAGGAGCTG (SEQ ID NO:20)
0137	CAGCTCCTCCGTGGATCCTGGACATTTCAG (SEQ ID NO:21)
0140	GACTGCGGCCGCTTTTTTTTTTTGAAATATTAAAAACAAAATCC (SEQ ID NO:22)
0220	CGAGAATCGATGCACTTCAGCC (SEQ ID NO:23)

Descriptions of oligonucleotides are as follows: 0077 introduces an *Apa*I endonuclease restriction site along with a serine to proline mutation within the coding region of nsP4. 0111 encodes an EcoRI endonuclease restriction site followed by the T7 promoter, a single G, and the first 18 nucleotides of the VEE IE genome. 0126 introduces a *Cla*I endonuclease restriction site within the coding region of nsP3 and was used in combination with 0220. 0113 was used to remove an *Apa*I endonuclease restriction site within the structural genes. 0140 encodes a unique NotI endonuclease restriction site followed by a 12 T's, and the reverse complement of the last 21 nucleotides of the VEE IE. A *Not*I site at the end of the cDNA encoding the VEE IE genome allowed for run-off transcription after digestion of the plasmid with *Not*I. 0220 introduces a *Cla*I endonuclease restriction site within the coding region of nsP3 and was used in combination with 0126. Minor alterations of the nucleotide sequence using 0077, 0113, 0126, and 0220 facilitated assembly of the full-length clones and allowed for rapid diagnostic analysis of virus generated from these clones by RT/PCR methods.

The full-length clone obtained, pIE1006, was transcribed *in vitro* using T7 polymerase of *Not*I-linearized plasmid and the RNA transfected into BHK-21 cells (Figure 4). The phenotype of the resulting virus was markedly different from the parent virus (strain 68U201) from which the cDNA was derived. The virus derived from pIE1006 *in vitro* transcribed RNA, VIE1006, gave rise to small plaques upon infection of target monolayers (Table 9). In attempts to recover the phenotypic characteristics of strain 68U201, regions of the pIE1006 clone were replaced with cDNA generated by RT-PCR from strain 68U201 RNA. Three

subsequent full-length, live clones were constructed by replacing pIE1006 sequences with 928, 4492, and 8130 nucleotides generated by RT-PCR of strain 68U201 RNA. These clones were designated pIE1007, pIE1008 and pIE1009; and viruses derived from these clones were designated VIE1007, VIE1008 and VIE1009, respectively. These clones are shown in Figure 4. The relevant cloning sites and genetic markers are indicated above each clone. Numbers indicate the corresponding nucleotides in the strain 68U201 genome. The T7 promoter is shown. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones. The characteristics of these viruses are described below.

EXAMPLE 6

Assessment of Viruses Derived from Various Clones. The viruses VIE1006, VIE1007, VIE1008 and VIE1009 were derived from plasmids pIE1006, pIE1007, pIE1008, and pIE1009, respectively. Analysis of virus derived from these clones by plaque assay is shown in Table 9. Plaque size was determined by infection of Vero cell monolayers followed by agarose overlay.

Analysis of virus derived from these clones (Table 9) by plaque assay indicated that VIE1007 gave the same plaque morphology as VIE1006 and therefore was not studied further. The VIE1008 produced larger plaques in comparison to VIE1006, and the VIE1009 virus gave rise to the largest sized plaques of the four viruses tested. Plaques generated by VIE1009 were similar to those produced by the parental virus, strain 68U201.

Table 9: In vitro analysis of virus derived from molecular clones of VEE IE strain 68U201

Virus Strain[1]	Plaque Size
VIE1006	small
VIE1007	small
VIE1008	medium
VIE1009	large
VEE IE 68U201	large

EXAMPLE 7

In Vivo studies of VEE IE Vaccine Candidates. The distinct plaque morphologies of VIE1006, VIE1008, and VIE1009 suggested that these viruses may behave differently *in vivo*. To assess the relative virulence of the cloned derivatives of strain 68U201, mice were initially inoculated with subcutaneously VIE1006, VIE1008, and VIE1009 viruses (Table 10). Mice infected with VIE1006 and VIE1008 were not adversely affected by these viruses as assayed by the number of mice surviving the infection. VIE1009 proved to be as virulent as strain 68U201, causing death in all of the animals infected (Table 10). Immunogenicity of the different viruses inferred by the demonstration of a protective immune response and was determined by back challenge of surviving animals in the virulence assay with approximately 10^4 pfu of the virulent, parental virus, strain 68U201. Back challenge was performed four weeks after the initial inoculation.

Table 10: In vivo analysis of virus derived from molecular clones of VEE IE strain 68U201

Virus Strain[1]	Mortality[2]	Challenge[3]
VIE1006	0/10	10/10
VIE1008	0/10	10/10
VIE1009	10/10	nd
VEE IE 68U201	nd	1/10

[1] Initial inoculation with approximately 10^4 PFU of each virus with the exception of VEE IE 68U201 which were left untreated until challenge phase of experiment.

[2] Expressed as animals dying/animals tested.

[3] Expressed as animals surviving/animals tested. All animals were challenged with approximately 10^4 PFU of VEE IE strain 68U201 four weeks after the initial inoculation.

EXAMPLE 8

Construction of a Full-length, Molecularly Attenuated VEE IE Clone. With the availability of a full-length virulent clone, specific attenuating mutations were introduced into the structural genes of the virus by site-directed mutagenesis. A deletion mutation was used instead of a point mutation because of the inability of viral RNA replication to repair such mutations. The glycoproteins of VEE IE are produced as a poly-protein precursor, PE2. The junction between the E3 and E2 proteins is cleaved by a furin-like cellular protease. The amino acid sequence of the presumed furin-like protease cleavage site of strain 68U201 is RGKR. The nucleotides encoding these four amino acids of the furin-like cleavage site between the E3 and E2

proteins were deleted from the pIE1009 clone and the resulting "cleavage deletion" clone was designated pIE1100. The oligonucleotides used to generate the cleavage deletion mutation are shown in Table 8. Oligonucleotide 0136 encodes a cleavage deletion mutation that eliminates the four amino acid furin-like cleavage site found in PE2 and was used in combination with oligonucleotide 0137. 0137 encodes the reverse complement of 0136.

Transfection of RNA transcribed from pIE1100 into tissue culture cells required extended incubation periods before viral cytopathic effect became apparent in the cultured cells. This extended incubation period is indicative of transcripts from full-length clones possessing mutations that partially inhibit viral replication. However, such mutations can be suppressed by second-site mutations which arise randomnly via the error prone process of alphavirus replication, resulting in variants with enhanced replication ability. In fact, the culture media from cells transfected with RNA transcribed from pIE1100 contained low titers of infectious virus, VIE1100, which could be amplified to high titer ($4x10^7$ PFU/ml) upon subsequent passage. Biochemical analysis showed that this virus had an uncleaved PE2 protein indicating that the cleavage deletion mutation totally prevented proteolytic processing of the surface E2 glycoprotein precursor. The efficacy of VIE1100 virus to serve as a vaccine was evaluated in mice as described below.

One specific suppressor mutation is thought to reside at nucleotide 10,181 of the VEE IE genome, a C to U nucleotide substitution resulting in an amino acid change from Serine to Leucine in the E1 protein positon #57. Ability of this mutation to suppress the

lethal nature of the furin cleavage site deletion mutation was assessed. The cDNA of pIE1100 was mutated by changing nucleotide 10,181 from a C to T. This introduced a serine to leucine change at amino acid number 57 of E1, a mutation found within the stock of VIE1100. The resulting clone was designated pVIE1150. Transfection of RNA from pVIE1150 into BHK-21 cells lead to the production of approximately 10^6 PFU/ml of virus in the supernatant of the transfected cells at 48 hours post-transfection.

EXAMPLE 9

Vaccine Studies with a Full-length, Molecularly Attenuated Virus. Balb/C mice were inoculated with VIE1100 virus at various doses (10^4, 10^6, and 10^7 PFU per mouse), and was found to be completely attenuated at a dose 100,000 times higher than that required to cause lethal disease by the parent virus, strain 68U201 (Table 10). Subsequent challenge of these animals with virulent strain 68U201 demonstrated that immunization with VIE1100 virus provided complete protection from lethal virus challenge (Table 11).

Table 11:Vaccination study with VIE1100

Virus Strain Dose	Mortality[1]	Challenge[2]
VIE1100		
10^4	10/10	10/10
10^6	10/10	10/10
10^7	10/10	10/10
Mock Vaccinated	0/10	0/10

1. Expressed as animals dying/animals tested.
2. Expressed as animals surviving/animals tested after inoculation with 10^4 PFU of VEE IE strain 68U201

EXAMPLE 10

Properties of a WEE-EEE chimeric virus. Based upon similarities of amino acid sequences of the carboxy terminal portion of NSP-4 and the amino terminal portion of the capsid proteins of WEE, EEE and Sindbis virus, it has been suggested that WEE virus arose by recombination between EEE and a Sindbis virus ancestor (Hahn *et al.* [1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**: 5997-6001). The capsid genes of both WEE and EEE contain a highly conserved sequence with a unique Bln I site 76 nucleotides downstream of the initiation codon. We utilized this site to insert the structural protein sequences of EEE into pWE5-18 in order to construct a full length clone encoding a chimeric virus. Plasmid pMWE-7 is a full length clone consisting of the 5′ non-coding sequence, nonstructural genes, 26S promoter and the first 25 codons for the capsid protein of WEE CBA/87 fused to the structural protein genes and 3′ non-coding sequence of EEE strain Fla91-4679. Transfection of BHK cells with RNA transcribed from pMWE-7 resulted in complete destruction of the monolayer and release of high yields of virus. SDS-polyacrylamide gel electrophoresis of the purified virus demonstrated that the virus is composed of polypeptides which comigrate with those of EEE and not of WEE, indicating that the virus is a chimera (Figure 3).

Injection of mice with the MWE chimeric virus killed mice only sporadically, suggesting that fusion of the sequences of the two viruses resulted in significant attenuation compared to the parent WE2000 virus (Table 12). Mice immunized with MWE-7 developed significant neutralizing antibody titers and resisted a lethal EEE Fla91-1467 challenge when immunized with greater than 10^5 plaque forming units of

MWE-7 virus. The neutralizing antibody response after challenge was not significantly elevated indicating that the immunization with the chimeric virus effectively prevented infection by a lethal EEE challenge. Therefore, a chimeric virus derived by combining the structural protein genes of EEE with the non-structural proteins genes of WEE may serve as a safe, effective approach to development of a vaccine for EEE virus.

Table 12. Immunization of C57BL6 Mice with an WEE/EEE Chimeric Virus Confers Protection Against a Lethal EEE Challenge

Virus Strain Dose	Mortality[1]	Mean Day to Death	Challenge[2]
WE2000			
10^5	10/10 (100)	8.5	
MWE7			
10^3	0/10 (0)		6/10 (60)
10^5	1/10 (10)	8.0	9/9 (100)
10^7	0/10 (0)[3]		9/9 (100)
Saline Control	0/10 (0)		0/10 (0)

1. Expressed as animals dying/animals tested
2. Expressed as animals surving/animals tested
3. One animal died during pre-challenge bleed

Example 12
VEE IIIA Chimera

Construction of IAB-IIIA cDNA Chimeric Clone (pV3A-1000)

BeAn8 (wild type Mucambo or VEE IIIA) cDNA was amplified with primers 128 and 140 (Table 13) to generate a PCR fragment of the entire 26S region of BeAn8. Primer 128 incorporated an ApaI site just upstream of the BeAn8 26S promoter to facilitate cloning into pV3000. Primer 140 introduced a NotI

site just downstream of the poly(A) tract which would also facilitate cloning into pV3000 as well as enable run-off transcription. This 128/140 PCR fragment was cloned into pBluescript SK+ vector using the ApaI and NotI sites and was termed pMUC-1000.

To replace the BeAn8 3' nontranslated region (3'NTR) in pMUC-1000 with the corresponding region in pV3000, an EcoRI site was introduced immediately downstream of the E1 stop codon in both pMUC-1000 and pV3000 using the primer pairs 013/227 and 225/226, respectively. The 013 primer is a universal primer found in the pBluescript SK+ vector and the 227 primer was designed to introduce an EcoRI site in pMUC-1000. The 225 primer introduced an EcoRI site in pV3000 and the 226 primer is located in the vector, TotoX. The 013/227 and 225/226 PCR fragments were separately cloned into the pBluescript SK+ vector producing pMUC-1200 and pV3nt-1000, respectively. The 3' NTR encoded in pV3nt-1000 was shuttled into pMUC-1200 using the EcoRI site and the NotI site to produce pMUC-1300.

The full-length chimeric cDNA clone, pV3A-1000, was constructed by shuttling the structural genes encoded in pMUC-1300 into the pV3000 nonstructural domain using the ApaI site and NotI site.

Table 13. Primers for construction of the pV3A-1000

013 -- AACAGCTATGACCATG (SEQ ID NO:24)
128 -- CTGAGAGGGGCCCCAGTAAC (SEQ ID NO:25)
140 -- GACTGCGGCCGCTTTTTTTTTTTTGAAATATTAAAAA (SEQ ID NO: 26)
225 -- CCAGAAACATAATTGAATTCAGCAGCAATTG (SEQ ID NO:27)
226 -- CTTTATCCGCCTCCATCC (SEQ ID NO:28)
227 -- CCAATCGCTGCTGAATTCTAATTATGTTTCTG (SEQ ID NO:29)

Five week old C57BL6 mice were used in a challenge study (Table 14). All mice immunized with

10^7 PFU of pV3A-1000 produced neutralizing antibodies. Mice were challenged with 10^7 PFU of 900807 (a Mucambo virus from Trinidad). The geometric mean neutralizing antibody titer in mice inoculated with 10^7, 10^5, and 10^3 PFU of pV3A-1000 was 9800, 8800, and 7000 respectively.

Table 14. Results from challenge studies

	Fraction of mice sick/inoc	Fraction of mice dead/inoc	Average days to onset of illness	Average days to death
Immunized with pV3A-1000	0/20	0/20	N/A	N/A
Unvaccinated	19/20	12/20	6.2	10.5

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11492 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:Double
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1 ATAGGGCATG GTATAGAGGC ACCTACCCTA CAAACAAATC GATCCAATAT

51 GGAAAGAATT CACGTTGACT TAGACGCTGA CAGCCCATAT GTCAAGTCGT

101 TACAGCGGAC GTTTCCACAA TTTGAGATCG AAGCAAGGCA GGTCACTGAC

151 AATGACCATG CCAATGCCAG AGCGTTTTCG CATGTGGCAA CAAAGCTCAT

201 TGAGAGCGAA GTCGACCGGG ACCAAGTTAT CTTGGACATT GGAAGTGCGC

251 CCGTCAGACA TGCACATTCC AACCACCGCT ATCATTGTAT CTGCCCCATG

301 ATAAGCGCTG AAGACCCGGA CAGACTACAG CGGTATGCAG AAAGACTTAA

351 GAGAAGTGAC ATGTACCGAC AAGAATATAG CCTCTNAAGG CGGCAGACCT

401 GCTGGAAGTC ATGTCCACAC CAGACGCAGA GACTCCATCT CTGTGTATGC

451 ACACAGACGC CACGTGTAGG TACTTTGGAA GTGTANGCAG TATACCAAGA

501 TGTGTACGCA GTCCATGCAC CGACATCAAT CTACCACCAG GCGCTTAAAG

551 GAGTTAGGAC AATTTACTGG ATAGGTTTTG ACACGACCCC TTTTATGTAC

601 AAAAACATGG CAGGTTCCTA CCCTACTTAC AACACAAACT GGGCCGACGA

651 GAGAGTATTG GAAGCACGTA ACATTGGCCT CGGTAACTCA GATCTTCAGG

701 AGAGCAGACT TGGAAAACTT TCAATCCTTA GGAAGAAGAG GCTCCAACCT

751 ACTGATAAGA TCATATTCTC GGTTGGTTCA ACAATCTACA CAGAGGATAG

801 ATCACTGTTA CGTAGCTGGC ATCTTCCAAA CGTGTTCCAC CTGAAAGGAA

851 AGTCTNACTT CACAGGTAGA TGTGGGACCA TTGTCAGCTG TGAAGGGTAT

901 GTCATCAAAA AGATAACGAT CAGCCCAGGA CTATACGGTA AAGTTGAGAA

951 CTTGGCGTCC ACGATGCATC GCGAGGGTTT CTTGAGTTGC AAAGTCACAG

1001 ATACGCTGCG CGGCGAGAGG GTTTCTTTTG CTGTGTGTAC GTATGTACCA

1051 GCCACACTTT GCGATCAGAT GACAGGGAGG GCTCAACCAA CGGATTGTCG

1101 TCAATGGTAG GACGCAAAGA AATACTNACA CAATGCAGAA CTATCTATTA

1151 CCAGTGGTCG CCCAGGCGTT TTCCAGGTGG GCGCGTGNAC ATCGTGCCGA

1201 CTTGGACGAC GAGAAGGAGC TAGGGGTGCG GGAGCGCACT CTTACTATGG

1251 GCTGCTGCTG GGCTTTCAAG ACCCAGAAAA TTACATCCAT CTACAAGAAG

1301 CCTGGTACGC AAACAAATTA AGAAAGTACC TGCCGTCTTT TGACTCATTT

1351 GTGATTCCGA CGCCTTACCA GCCACGCGGG GGCTCGAATA TGGGCCTTCC

1401 GCCGTNAGGC TCAAGCTGCT GCTTGAACCA ACTGTCAAAC CCGCACCGGC

1451 TATTACAATG GCCGATGTGG AGCACCTGCG TGGCTTACAG CAAGAAGCTG

1501 AAGAAGTGGC TGCAGCGGGA AGAGATCAGA GAAGCCCTGC CACCCTTGCT

1551 CCCTGAAATA GAAAAAGAGA CCGTAGAGGC AGAAGTAGAC CTCATTATGC

1601 AAGAGGCAGG AGCAGGTAGC GTGGAGACAC CNACGAGGAC ATATCAAGGT

1651 AACAAGTTAC CCAGGTGNAA GAGAAGATTG GGTCTTATCC CTATACTTTC

1701 ACCCCAGGCG GTTTTANAAT NGTNAAAAAC TGGCGTGTAT CCACCCATTG

1751 GCGGAACAAG TACTGGTAAT GACTCACAAA GGCAGGGCCG GGCGATACAA

1801 AGTCGAGCCA TACCACGGTA NGGTCATTGT ACCCAGAAGG GACGGCGGGT

1851 CCCTGTTCAA GACTTTCAGG CACTGAGTGA GAGCGCCACG ATCGTTTTCA

1901 ACGAGAGGGA GTTCGTAAAC CAGATATTTT GCACCCACAT CGCAAGCKTT

1951 TCAACTATAG TGAGTCGCTA TTACACTGAC GAAGAGTACT ATAAGATTGT

2001 AAAGACTCAG GACGCAGACT CAGAATACGT CTTTGACATT GACGCACGAA

2051 AGTGTGTTAA GCGAGAAGAC GCAGGTCCCT TGTGCCTAAC TGGTGATCTG

2101 GTAGATCCAC CATTTCACGA GTTTGCGTAC GAGAGTCTCA AGACACGACC

2151 AGCAGCACCT CACAAAGTCC CAACCATCGG AGTCTATGGA GTGCCAGGTT

2201 CAGGTAAATC TGGGATCATC AAAAGCGCTG TGACTAAGAA AGATCTGGTT

2251 GTGAGTGCGA AGAAGGAAAA CTGCGCAGAA ATTATCAGGG ATGTAAGGAG

2301 GATGAGACGT ATGGATGTTG CTGCTAGGAC TGTTGATTCA GTGCTTCTAA

2351 ATGGGGTTAA GCACCCCGTT AACACCCTGT ACATTGATGA GGCATTTGCC

2401 TGCCATGCAG GGACGCTGCT GGCACTGATT GCCATCGTCA AACCTAAGAA

2451 AGTGGTATTG TGCGGGGACC CAAAACAATG CGGCTTCTTT AACATGATGT

2501 GCCTGAAAGT ACATTTTAAC CATGACATAT GCACTGAGGT GTACCACAAA

2551 AGCATCTCTA GGAGGTGCAC ACAGACTGTA ACCGCCATTG TCTCCACGCT

2601 CTTCTACGAC AAGCGAATGA AGACGGTTAA CCCATGTGCT GACAAAATCA

2651 TCATAGATAC CACAGGGACC ACAAAGCCGC ACAAAGATGA TCTGATTCTA

2701 ACCTGTTTCA GAGGATGGGT GAAACAGCTA CAGATTGACT ATAAAAATCA

2751 TGAAATCATG ACTGCGGCTG CATCGCAAGG ACTTACGCGG AAAGGCGTTT

2801 ATGCTGTCAG GTACAAAGTC AACGAGAATC CACTCTACTC GCAGACTTCT

2851 GAGCACGTGA ACGTGTTAGT TACACGCACA GAAAAACGCA TTGTCTGGAA

2901 GACGCTAGCT GGTGACCCCT GGATAAAGAC ACTTACAGCT AAATACTCCG

2951 GGGATTTCAC GGCTTCATTG GACGACTGGC AACGCGAACA CGACGCTATT

3001 ATGGCACGCG TTCTTGATAA GCCGCAGACA GCTGATGTGT TCCAGAATAA

3051 GGTGAACGTC TGCTGGGCGA AGGCTTTAGA GCCAGTCTTG GCCACGGCCA

3101 ACATTGTGCT GACGAGACAG CAGTGGGAGA CGTTGCACCC ATTCAAGCAT

3151 GACAGAGCGT ACTCACCTGA AATGGCACTG AACTTCTTTT GCACCAGGTT

3201 CTTTGGTGTA GACCTGGACA GTGGGTTGTT TTCCGCTCCT ACCGTCGCAC

3251 TTACTTACAG GGATCAGCAC TGGGACAACT CGCCAGGGAA GAACATGTAT

3301 GGGCTTAATA GAGAGGTAGC AAAGGAGCTG TCACGGCGAT ATCCGTGCAT

3351 CACAAAAGCG GTTGACACAG GCAGGGTAGC TGATATAAGG AATAATACCA

3401 TCAAGGACTA CTCTCCAACA ATTAATGTGG TTCCATTAAA TCGCCGGTTG

3451 CCCCACTCGC TGATCGTTGA CCACAAAGGA CAGGGTACAA CTGATCACAG

3501 TGGATTCCTA TCTAAGATGA ATGGCAAATC TGTGTTGGTG ATCGGCGATC

3551 CTATCAGCAT TCCTGGGAAG AAAGTAGAGT CCATGGGTCC ATTGCCCACT

3601 AATACCATCA GGTGTGATCT CGATTTGGGA ATACCTAGCC ATGTCGGTAA

3651 ATATGACATT ATATTTGTCA ATGTTAGGAC CCCATACAAG AACCATCACT

3701 ACCAACAGTG CGAGGATCAC GCTATCCACC ACAGCATGCT AACGTGTAAG

3751 GCTGTCCACC ACCTGAACAC TGGCGGAACA TGTGTGGCCA TAGGGTATGG

3801 ACTTGCTGAT CGCGCAACCG AGAATATCAT CACTGCGGTG GCTCGCTCAT

3851 TTAGGTTTAC CCGTGTCTGT CAGCCTAAGA ACACTGCCGA AAATACTGAG

3901 GTTCTCTTCG TGTTCTTCGG CAAGGACAAC GGCAACCACA CACATGACCA

3951 GGACAGACTC GGTGTAGTGC TTGACAACAT CTACCAAGGG TCAACCAGGT

4001 ACGAGGCAGG GAGAGCTCCA GCGTACAGAG TGATCAGAGG TGACATTAGC

4051 AAGAGCGCTG ACCAAGCTAT CGTTAATGCT GCTAATAGCA AAGGTCAACC

4101 AGGTTCCGGA GTGTGCGGTG CACTGTACCG AAAATGGCCG GCTGCTTTTG

4151 ATAGACAGCC AATAGCTGTC GGGACGGCTA GACTTGTGAA GCACGAACCG

4201 CTCATCATAC ATGCTGTAGG ACCGAATTTT TCTAAGATGC CGGAACGAGA

4251 GGGCGACCTT AAGCTCGCAG CTGCCTACAT GAGCATAGCG TCCATCGTCA

4301 ACGCTGAGCG GATTACTAAA ATATCAGTAC CGCTACTGTC AACTGGCATC

4351 TATTCTGGTG GCAAAGATCG AGTGATGCAA TCATTGCATC ACTTGTTCAC

4401 TGCTTTCGAC ACTACGGATG CCGATGTCAC CATATATTGC TTGGATAAAC

4451 AATGGGAGAC CAGGATAACC GAGGCCATTC ACCGCAAAGA AAGCGTCGAA

4501 ATACTGGATG ATGATGACAA GCCAGTAGAC ATCGACTTGG TCAGGGTCCA

4551 CCCAAACAGC TCTTTGGCAG GCAGACCAGG TTACTCCGTC AATGAGGGCA

4601 AGCTGTATTC ATACCTGGAA GGTACACGAT TCCATCAGAC CGCCAAGGAC

4651 ATTGCCGAAA TCCATGCAAT GTGGCCCAAC AAATCTGAGG CTAATGAGCA

4701 GATTTGCTTG TACATCCTGG GGGAGAGTAT GTCCAGCATC CGCTCCAAAT

4751 GCCCAGTAGA GGAGTCAGAG GCGTCTGCTC CACCTCACAC ACTTCCATGC

4801 CTGTGTAATT ACGCTATGAC GGCTGAGCGC GTATACAGGT TGCGCTCTGC

4851 GAAGAAAGAA CAGTTCGCCG TATGCTCATC ATTCCCGTTG CCGAAGTACA

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7601 AGGTCGATAG CCAACTTAAC TTTCAAACAA CGATCACCTA ATCCGCCGCC

7651 AGGTCCGCCG CCGAAGAAGA AGAAGAGTGC TCCCAAGCCA AAACCTACTC

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7751 CCAGGGAAAC GACAGCGTAT GTGTATGAAG TTGGAGTCGG ACAAGACATT

7801 TCCGATCATG TTGAACGGCC AAGTGAATGG ATACGCTTGC GTTGTCGGAG

7851 GAAGGCTGAT GAAACCACTC CACGTTGAAG GAAAAATCGA TAATGAGCAA

7901 TTAGCGGCCG TGAAATTGAA GAAGGCTAGC ATGTACGACC TGGAGTATGG

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9651 CAGCATTAGC AGTTTTGTGC TGTATTCGGC CAACCAACGC TGAAACATTT

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9851 GACGCCTTCG AACATGCGAC CACTGTGCCA AATGTTCCGG GGATCCCGTA

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9951 CGGTCGTCTC ATCGGAATTA ACACCCTCAA CTAACAAGGA GTACGTGACC

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10551 CCTACACCCA AGCAGTATCA GGGTATGAAA TGTGGAAGAA CAACTCAGGA

10601 CGACCCCTGC AAGAAACAGC ACCATTCGGA TGTAAAATTG AAGTGGAGCC

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10701 TCCCTGATGC AGCTTTTGTG AGATCATCTG AATCACCAAC AATTTTAGAA

10751 GTCAGCTGCA CAGTAGCAGA CTGCATTTAT TCTGCAGACT TTGGTGGTTC

10801 GCTAACACTA CAGTACAAAG CTAACAGAGA GGGACATTGT CCAGTTCACT

10851 CCCACTCCAC TACAGCTGTT TTGAAGGAAG CGACCACACA TGTGACTGCC

10901 ACAGCCATAA CACTACATTT TAGCACATCG AGCCCACAAG CAAATTTCAT

10951 AGTTTCGCTA TGGCGCAAGA AGACCACCTG CAATGCTGAA TGTAAACCAC

11001 CGGCCGACCA CATAATTGGA GAACCACATA AGGTCGACCA AGAATTCCAG

11051 GCGGCAGTTT CCAAAACATC TTGGAACTGG CTGCTTGCAC TGTTTGGGGG

11101 AGCATCATCC CTCATTGTTG TAGGACTTAT AGTGTTGGTC TGCAGCTCTA

11151 TGCTTATAAA CACACGTAGA TGACTGAGCG CGGACACTGA CATAGCGGTA

11201 AAAACTCGAT GTACTTCCGA GGAAGCGTGG TGCATAATGC CACGCGCCGC

11251 TTGACACTAA AACTCGATGT ATTTCCGAGG AAGCACAGTG CATAATGCTG

11301 TGCAGTGTCA CATTAATCGT ATATTACACT ACATATTAAC AACACTATAT

11351 CACTTTTATG AGACTCACTA TGGGTTTCTA ATACACACTA CACACATTTT

11401 ATTTAAAAAC ACTACACACA CTTTATAAAT TCTTTTATAA TTTTTCTTTT

11451 GTTTTTTATT TTGTTTTTAA AATTTCAAAA AAAAAAAAAA AA

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11464 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:Double
(D) TOPOLOGY: Unknown

1 ATGGGCGGCG CATGAGAGAA GCCCAAACCA ATAACTACCC AAAATGGAGA AAGTTCACGT

61 TGACATCGAG GAAGATAGTC CCTTCCTCAG AGCATTACAA CGGAGCTTCC CGCAGTTTGA

121 GGTAGAAGCC AAGCAGGTCA CAGATAATGA CCATGCTAAC GCCAGAGCGT TTTCGCATTT

181 GGCATCGAAA TTGATCGAGA CGGAGGTGGA ACCATCCGAT ACGATCCTAG ACATTGGAAG

241 TGCGCCTGCC CGCAGAATGT ATTCCAAGCA TAAGTACCAT TGCATCTGTC CGATGAAATG

301 TGCAGAAGAT CCGGACAGAC TGTTTAAGTA TGCAGCCAAG CTGAAGAAGA ACTGTAAAGA

361 GATTACAGAT AAGGAACTGG ACAAGAAGAT GAAGGAGCTT GCGGAAGTCA TGAGCGACCC

421 TGATCTCGAA ACTGAAACGA TTTGCCTTCA CGACGATGAA ACCTGTCGAT TTGAGGGTCA

481 AGTCGCAGTG TATCAGGATG TGTACGCGGT TGACGGACCG ACGAGCCTTT ACCATCAGGC

541 CAACAAAGGG GTCAGAGTCG CCTATTGGAT AGGATTCGAC ACTACCCCTT TTATGTTTAA

601 GAACCTGGCT GGAGCATATC CCTCCTATTC GACCAACTGG GCCGACGAGA CCGTGTTAAC

661 GGCTCGTAAT ATAGGCTTGT GCAGCTCCGA TGTGATGGAG CGGTCACGTA GAGGGATGTC

721 CATCCTCAGA AAGAAATTTT TAAAACCATC CAATAACGTC TTGTTCTCTG TAGGATCTAC

781 CATCTACCAC GAGAAGCGAG ACTTACTAAG GAGTTGGCAC CTACCGTCCG TTTTTCACCT

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961 GATGCATCGC GAGGGATTCT TGTGCTGCAA GGTGACGGAC ACGCTTGACG GGGAGAGGGT

1021 CTCTTTCCCC GTATGCACGT ACGTGCCAGC CACATTGTGC GACCAAATGA CAGGCATTCT

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1381 TTTCCACTCT TTCGTGCTGC CCAGAATTGG AAGCAACACC TTGGAGATTG GGCTGAGGAC

1441 CAGGATCAGA AAACTACTGG AGGAACCTGT GGACAGACCA CCACTGATTA CCGCCGACGA

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2581 GTGTACCAAG TCAGTGACGT CGGTAGTGTC CACACTGTTT TATGACAAAA GAATGAGAAC

2641 TACTAACCCA AGGGATTCCA AAATCGAAAT TGACACAACA GGAAGTACTA AACCAAAAAA

2701 GGATGACTTG ATTCTCACAT GTTTTAGGGG ATGGGTTAAG CAACTGCAAA TAGACTACAA

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3361 TCAATTACCA CGTGCGGTGA CTACTGGGAG AGCATACGAC ATGAACACCG GTACTTTGCG

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6421 GTATTGGGAG ACCTACAAGA AGAATCCTAT TAGATTGACC GAGGAAAATG TGGTCAACTA

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6721 AGTTCTGTTG CCCAACATAC ACACATTATT TGACATGTCT GCTGAGGATT TCGACGCCAT

6781 AATTGCCGAG CACTTCCAAC CAGGCGATTG GGTGTTAGAG ACAGACATAG CGTCATTCGA

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7021 GTTTGTCAAT ACTGTGATCA ATATGGTCAT AGCTAGCAGA GTTCTGCGTG AGAGACTGAC

7081 AAACTCCCCT TGCGCCGCGT TCATTGGCGA CGACAATATC GTGAAAGGGG TTAAGTCCGA

7141 CAAACTCATG GCCGATAGGT GCGCTACATG GTTGAACATG GAAGTCAAAA TCATCGACGC

7201 AGTGGTTGGC GAGAAAGCTC CCTACTTCTG TGGTGGGTTT ATTTTATGTG ACTCTGTGAC

7261 CGGAACTGCA TGCCGTGTAG CAGACCCTTT GAAGAGATTA TTTAAGCTTG GAAAACCACT

7321 GGCTGTGGAT GATGAACATG ATGATGACAG GCGTCGAGCA CTACAGGAGG AATCTGCCCG

7381 GTGGAACCGG GTGGGAATTT TTTCCGAGCT GTGCAAAGCC GTCGAGTCGC GATATGAAAC

7441 AGTGGGCACG GCTGTCATTA TCATGGCCAT GACTACGCTC GCCAGCAGTG TCGAGTCGTT

7501 CAGTTGTCTA AGAGGGGCTT CTATATCCCT CTACGGCTAA CCTGAATGGA CTGCGACGTA

7561 GTCAAGTCCG CCGAAATGTT TCCTTATCAA CCAATGTACC CAATGCAGCC CATGCCCTTC

7621 CGCAACCCTT TTGCGACTCC CAGAAGACCA TGGTTTCCAA GGACCGACCC CTTTTTAGCG

7681 ATGCAGGTGC AAGAGCTGGC AAGGTCCATG GCCAACTTGA CGTTCAAGCA ACGGCGAGAT

7741 GTGCCGCCCG AGGGTCCACC GGCTAAGAAG AAGAAGAAGG ACAACTCGCA ACAAGGTGGT

7801 CGGAATCAGA ATGGAAAGAA AAAGAACAAG CTAGTAAAGA AAAAGAAGAA GACAGGGCCA

7861 CCACCCCCAA AAAATACTGG TGGCAAAAAG AAAGTCAATA GGAAGCCAGG GAAGAGACAA

7921 CGAATGGTTA TGAAGTTGGA GTCAGACAAG ACATTCCCTA TCATGCTAGA TGGAAAAGTT

7981 AATGGATATG CATGCGTGGT CGGTGGCAAG CTGTTTAGAC CACTGCATGT GGAGGGTAAG

8041 ATTGACAATG ACGTGTTGTC CTCCCTCAAG ACCAAAAAGG CATCTAAGTA TGATCTGGAG

8101 TATGCTGATG TGCCGCAGAG CATGCGCGCA GACACATTTA AATACACTCA TGAAAAACCC

8161 CAGGGCTATT ACAGCTGGCA CCATGGAGCA GTACAGTATG AAAATGGCAG ATTCACAGTG

8221 CCCAAAGGAG TCGGAGCCAA AGGAGATAGC GGTCGCCCCA TACTTGACAA CCAAGGGCGT

8281 GTGGTCGCTA TTGTGCTTGG CGGAGTGAAT GAAGGCTCCA GAACGGCACT GTCTGTCGTG

8341 ACGTGGAACG AAAAAGGGGT TACAGTCAAA TACACCCCCG AGAATAGCGA GCAGTGGTCC

8401 CTGGTGACCA CCATGTGCCT GCTAGCCAAT GTCACGTTCC CGTGCACCCA ACCACCCATC

8461 TGCTACGACC GTAAGCCAGC AGAGACTTTG TCCATGCTCA GTCATAACAT AGACAATCCT

8521 GGTTATGACG AGTTGCTCGA AGCAGTACTG AAATGTCCAG GCAGAGGCAA GAGGTCCACG

8581 GAGGAGCTGT TTAAGGAGTA CAAGTTAACA CGCCCGTACA TGGCCAGGTG CATCAGGTGT

8641 GCGGTCGGAA GTTGCCACAG CCCCATAGCC ATAGAGGCGG TAAGGAGCGA AGGGCACGAT

8701 GGCTATGTAC GACTCCAGAC CTCATCTCAG TATGGATTAG ACCCATCAGG AAACTTGAAA

8761 GGCAGAACCA TGAGGTATGA TATGCATGGA ACCATAGAAG AGATACCGTT GCATCAGGTG

8821 TCTGTTCATA CCTCACGTCC TTGCCACATA ATAGATGGGC ATGGATACTT TCTGCTTGCC

8881 AGGTGCCCTG CAGGAGACTC CATAACTATG GAATTTAAGA AAGAATCAGT CACCCATTCC

8941 TGCTCTGTGC CCTACGAAGT AAAGTTTAAT CCTGCGGGAA GAGAACTGTA CACACACCCA

9001 CCAGAGCACG GAGCTGAACA ACCTTGTCAC GTGTACGCTC ACGACGCACA AAATAGGGGA

9061 GCTTACGTGG AAATGCACCT TCCAGGATCC GAAGTGGACA GTACTTTACT GTCCATGAGC

9121 GGTAGTTCTG TTCATGTGAC TCCACCTGCC GGGCAAAGCG TCCAAGTGGA ATGCGAATGT

9181 GGTGGCACCA AGATCTCTGA AACCATCAAT TCAGCTAAAC AATACAGTCA GTGCTCAAAG

9241 ACATCTCAGT GCAGGGCATA CCGTACACAG AATGACAAAT GGGTGTACAA TTCGGATAAA

9301 CTGCCTAAAG CATCGGGAGA AACTCTCAAA GGCAAATTGC ATGTGCCCTT CGTACTGACC

9361 GAAGCGAAAT GCACAGTACC ATTGGCTCCA GAACCCATTA TCACCTTTGG GTTCCGCTCT

9421 GTGTCTCTGA AACTTCATCC TAAGAACCCC ACCTTCCTAA CCACGAGGCA GCTGGATGGA

9481 GAACCAGCTT ACACCCACGA ACTTATAACC CACCCTGTGG TGAGAAATTT CTCGGTTACA

9541 GAGAAAGGTT GGGAATTTGT GTGGGGAAAC CATCCGCCTC AAAGGTACTG GTCTCAAGAA

9601 ACTGCACCAG GTAATCCACA CGGACTACCA CACGAGGTGA TCACGCATTA CTATCACAGA

9661 TATCCCATGT CCACCATCCT CGGCTTATCA ATCTGTGCGG CGATAGTGAC GACATCCATT

9721 GCGGCATCCG TATGGCTGTT TTGCAAATCA CGGATTTCAT GCCTGACCCC CTATCGCTTG

9781 ACTCCGAATG CCAGCATGCC TCTGTGCTTA GCCGTCTTGT GCTGCGCACG CACAGCCAAA

9841 GCCGAAACTA CTTGGGAATC CCTAGATCAC CTCTGGAACC ACAACCAGCA GATGTTCTGG

9901 AGTCAGCTGC TAATCCCGCT AGCAGCACTG ATAGTTGCTA CCCGCTTGCT GAAATGTGTG

9961 TGTTGCGTTG TGCCTTTTTT AGTCGTGGCC GGCGCCGTAG GCGCCGGCGC TTACGAGCAC

10021 GCGACTACGA TGCCGAACCA AGTGGGGATC CCGTATAATA CCATTGTCAA CAGAGCGGGT

10081 TATGCACCTC TACCTATTAG CATAGTACCT ACTAAAGTGA AGCTGATTCC AACAGTGAAT

10141 CTTGAGTACA TTACATGCCA TTACAAGACT GGAATGGATT CACCCGCCAT TAAATGCTGC

10201 GGCACTCAGG AGTGTTCTCC AACTTACAGG CCGGACGAGC AATGCAAAGT CTTCTCTGGA

10261 GTATACCCAT TTATGTGGGG AGGGGCGTAT TGCTTTTGCG ATACGGAGAA TACCCAGATA

10321 AGCAAGGCGT ACGTGACGAA ATCGGAAGAT TGCGTCACCG ATCACGCCCA GGCATACAAA

10381 GCACATACAG CCTCAGTCCA AGCCTTCTTA AATATTACAG TTGGAGGACA CTCAACGACA

10441 GCAGTGGTGT ATGTGAATGG AGAGACTCCC GTTAATTTTA ATGGAGTGAA GCTGACCGCG

10501 GGCCCTCTGT CCACAGCCTG GTCGCCGTTC GACAAGAAGA TCGTGCAGTA CGCCGGGGAA

10561 ATTTATAACT ATGACTTTCC GGAATATGGA GCCGGCCACG CAGGAGCGTT TGGTGACATC

10621 CAGGCTAGGA CGGTATCTAG TTCCGATGTA TACGCCAACA CAAACCTTGT GCTGCAGAGA

10681 CCCAAAGCCG GAGCGATCCA TGTCCCGTAC ACCCAGGCCC CATCTGGGTA TGAACAATGG

10741 AAGAAAGATA AACCACCATC CCTCAAGTTC ACAGCCCCGT TCGGTTGTGA AATTTACACC

10801 AACCCTATCC GTGCTGAAAA CTGCGCTGTG GGATCAATTC CGCTAGCTTT TGACATTCCC

10861 GATGCTCTGT TTACCAGGGT GTCCGAAACA CCGACATTAT CTGCTGCCGA GTGCACTCTG

10921 AACGAGTGTG TATATTCATC CGACTTTGGC GGGATCGCTA CAGTCAAATA CTCGGCGAGC

10981 AAGTCAGGCA AATGTGCAGT TCATGTACCC TCAGGCACGG CTACATTGAA AGAAGCCGCA

11041 GTCGAGTTGG CCGAACAGGG TTCGGCTACT ATACATTTTT CGACTGCCAG CATTCATCCG

11101 GAGTTTAGAC TCCAGATATG CACGTCTTAC GTTACGTGCA AAGGGGATTG TCACCCTCCG

11161 AAAGATCACA TTGTGACGCA TCCCCAATAC CACGCCCAGT CATTTACAGC TGCGGTATCA

11221 AAAACCGCTT GGACGTGGTT AACATCCTTA CTGGGAGGGT CAGCTATAAT TATAATAATT

11281 GGACTTGTGT TAGCCACAGT TGTGGCTATG TATGTGCTGA CCAACCAGAA ACATAATTAG

11341 TATTAGCAGC GATTGGCATG CTGCTTGTAA AGTTTTATTA CAAATAACGT GCGGCAATTG

11401 GCGAGCCGCT TTAATTAGAA TTTTATTTTC TTTTACCATA ATTGGATTTT GTTTTTAATA

11461 TTTC

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4003 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:Double
(D) TOPOLOGY: Unknown

Nucleotide Sequence of the BeAn8 26S Subgenomic cDNA containing last portion of NS4, the 26S promoter, the complete BeAn8 structural genes and a portion of the 3' noncoding region.

1 CCCAGTAACT CTCTACGGCT GACCTGAATG GACTGTAACG TAGTTCAGTC

51 CGCAACCATG TTCCCTTACC AATCACCAAT GTTTCCAATG CAACCAGCGC

101 CTTTTCGCAA CCCGTACGCT CCTCCTAGAA GACCGTGGTT CCCTAGAACC

151 GATCCCTTCT TAGCCATGCA GGTGCAGGAG TTGGCCCGAT CAATGGCGAG

201 CTTGACGTTT AAACAGCGTC GAGATACGCC ACCCGAGGGG CCACCTGCTA

251 AGAAGAAGCG TAAGGAGCCT CAACAGCAGG TAGCTCAGGC GCAGGTTAAG

301 AAAAAGAACG GAAAACCGAA GAAGAAGAAA AGTAACGGAG CACCACCCCC

351 AAAAAATCAG AAGAGCACCA AGAAGAAGAC CAATAAGAAA CCTGGAAAAA

401 GACAACGGAT GGTTATGAAG TTAGAATCAG ACAAAACATT TCCTATTTTG

451 CTGGATGGAA AAATTAATGG ATATGCTTGC GTTGTTGGAG GAAAGCTATT

501 TCGACCCATG CACGTGGAAG GCAAAATTGA CAATGAAACT CTTGCCTCCC

551 TGAAGACGAA GAAGGCATCC AAATACGACC TAGAGTATGC CGATGTTCCT

601 CAAAGCATGC GAGCAGATAC CTTTAAATAC ACACATGATA AGCCTCAAGG

651 GTATTATAAT TGGCATCACG GCGCCGTGCA GTATGAAAAT GGGAGATTCA

701 CGGTGCCGAA AGGTGTGGGA GCGAAAGGAG ACAGTGGACG CCCCATACTA

751 GATAATCAAG GCAGAGTCGT GGCCATTGTG CTGGGAGGGG TGAATGAAGG

801 ATCCAAGACA GCTTTGTCCG TAGTTATGTG GAATGAAAAA GGGGTCACCG

851 TAAAATATAC ACCAGAAAAC TGTGAGCAAT GGTCACTAGT TACCACCATG

901 TGTCTTCTCG CCGATGTTAC GTTCCCTTGT TCCACTCCAC CAATTTGCTA

951 CGACCGAGCA CCCGCAGAAA CCCTGATGAT GCTTAGCAAG AACATTGACA

1001 ATCCTGGCTA TGATGAATTG CTGGAAGCAG TGCTGAAATG CCCCGGCAGA

1051 CAGAAGAGAT CTACGGAGGA GTTATTTAAG GAGTACAAAC TTACACGTCC

1101 GTACATGGCC AAGTGTGTGC GGTGTGCCGT TGGAAGTTGC CACAGCCCGA

1151 TCGCTATAGA AGCAGTAAGA AGCGACGGGC ATGACGGCTA CATCCGAATA

1201 CAGACATCAT CACAGTACGG TTTAGACCCC TCCGGGAACG TTAAGAGCAG

1251 AGTTATGAGG TATAATCTGT ATGGCAAGAT CGTAGAAGTT CCATTACATC

1301 AGGTTTCATT ACACACATCT CGGCCTTGCC ACATTATTGA TGGTCACGGA

1351 TATTTCCTCC TCGCACGCTG CCCAGAGGGC GACTCTATCA CCATGGAGTT

1401 TAAGAAGGAT TCCGTCACCC ATTCCTGTTC AGTGCCTTAT GAAGTGAAAT

1451 TCACACCCGT GGGCAGAGAA TTATATAGCC ATCCCCCAGA ACACGGCACA

1501 GAACATCCGT GCCGTGTGTA TGCCCACGAC GCCCAGCAAA AAGATGCGTA

1551 TGTGGAGATG CACCTGCCCG GGTCCGAAGT TGACAGTTCC CTGCTCTCCA

1601 TGAGCGGTAG TGCGGTCCGG GTAACACCAC CATCAGGGCA AAGTGTCCTT

1651 GTGGAGTGCA ACTGTGGCTC CGCTGTGTCG GAAACCATAA ACACTGCAAA

1701 ATCATACAGC CAATGCACAA AAACATCACA ATGCCGCGCG TACCGTCTGC

1751 AGAATGATAA GTGGGTATAC AATTCAGACA AACTTCCAAA GGCATCGGGA

1801 GAAACGCTGA AAGGGAAACT GCATGTACCT TATCTCCTTT CCGAAGCGAA

1851 GTGTACCGTA CCTTTAGCAC CCGAGCCAAT AGTAACCTTC GGCTTTCGAT

1901 TCGTATCTTT GAAATTGCAT CCACGGAATC CGACATATTT GACTACCCGC

1951 CAGCTAGATG GAGAACCGAA TTACACCCAC GAGCTAATTT CAGAGCCAAC

2001 AACTAGAAAT TTTACAGTGA CTGAGCATGG ATGGGGATAC GTTTGGGGTA

2051 ATCACCCGCC TCAGAGGTAC TGGGCACAGG AGACAGCTCC AGGCAACCCG

2101 CATGGGCTGC CGCACGAGGT GATTACTCAT TACTATAACA GGTACCCAAT

2151 GTCCACGATT TTCGGACTAT CGATTTGCGC CGCAGTGGTA ACCACCTCAA

2201 TAGCCGCATC CACCTGGCTG TTGTGCAAGT CGAGAGTATC TTGTTTGACT

2251 CCGTATCGAC TGACCCCGAA TGCTCAGTTA CCTGTGTGTC TAGCCTTCCT

2301 GTGCTGCGCG AGGACAGCCC GTGCAGAGAC CACATGGGAA TCACTAGACC

2351 ATTTATGGAA CCATAATCAA CAGATGTTCT GGAGTCAACT GCTCATTCCC

2401 CTAGCCGCGC TCATTGTGGT GACCCGCTTG CTGAAGTGCA TGTGTTGCGT

2451 CGTTCCTTTT TTAGTCCTAG CAGGCGCCGC AAGCGTCGGC GCCTACGAAC

2501 ACGCAACCAC GATGCCGAAT CAGGTGGGGA TCCCGTATAA TACAGTAGTC

2551 AACCGCGCAG GTGACGCACC ATTGGCAATC AGCATTATTC CAACCAAGAT

2601 ACGGCTAATT CCTACTTTGA ATTTAGAATA TATTACATGC CACTATAAGA

2651 CAGGATTAGA TTCACCTTTC ATTAAATGCT GCGGAACGCA GGAATGCCCC

2701 CAAGTGAATA GACCCGATGA ACAGTGTAAA GTCTTCACGG GGGTGTATCC

2751 GTTTATGTGG GGAGGCGCCT ACTGCTTCTG TGACTCTGAA AACACGCAAA

2801 TTAGTCGAGC GTATGTGATG AAATCAGATG ACTGCTCAGC TGACCACGCC

2851 TTGGCCTACA AAGCTCATAC TGCCTCAGTC CAAGCTTTTC TGAATATAAC

2901 TGTGGGAGAG CAATCGACGA CAGCGGTAGT GTACGTGAAT GGAGAAACAC

2951 CGGTCAATTT TAACGGCATT AAATTGGTTG CAGGCCCTTT ATCAACTGCC

3001 TGGACCCCAT TTGATCGGAA AGTGGTGCAG TACGCCGGAG AGATCTACAA

3051 TTATGACTTC CCGGAGTACG GAGCTGGGCA TGCAGGGGCG TTCGGGGATC

3101 TTCAAGCCAG AACAATCACC AGTAATGACC TGTACGCCAA CACGAATTTA

3151 GTATTGCAAA GACCCAACAC AGGCACCATC CATGTTCCTT ACACGCAGGC

3201 ACCGTCAGGC TTTGAGCAGT GGAAGAAAGA CAAACCACCA TCATTAAAGT

3251 ACACCGCACC ATTTGGGTGC GAAATTCATG TGAATCCCGT CAGAGCTGAG

3301 AATTGCGCAG TAGGATTTAT ACCATTAGCC TTCGACATAC CCGATGCCTT

3351 GTTTACCAGG GTGTCAGAAA CACCGACGTT GTCGAGCGCT GAGTGCTCCT

3401 TGAATGAGTG TACATACTCA ACGGACTTTG GCGGGATCGC CACTGTCAAG

3451 TACTCGGCTA GTAAATCAGG CAAATGCGCA GTACATGTTC CCTCAGGCAC

3501 TGCAACTCTG AAAGAGTCAT TGGTGGAAGT GGTCGAACAA GGGTCAATGA

3551 CCCTTCACTT TTCGACCGCC AGTATACACC CAGAGTTTAA ATTGCAGATC

3601 TGTACGAAGG TACTCACATG TAAAGGCGAC TGTCATCCGC CTAAAGACCA

3651 TATTGTGACG CACCCCCAGC ACCACGCCCA GACATTTACA GCTGCGGTAT

3701 CCAAGACCGC TTGGACGTGG TTAACGTCAC TCTTAGGAGG GTCAGCAGTA

3751 ATTATTATAA TTGGCCTTGT ATTAGCAACT GTTGTTGCTA TGTATGTGCT

3801 GACCAACCAG AAACATAATT AGACCACAGC AGCGATTGGA AAGCTGCCTA

3851 TTAGAAACAT GTAGCGGCAA TTGGCAAGCC GCCTATAAAT GTTTAGCAGC

3901 AATTGGCAAG CTGCATATAT AAATTACCTA GCGGCAATTG GCACGCCGCT

3951 TATAAAATTT TTATTTTCTT TTACCAATAA TTGGATTTTG TTTTTAATAT

4001 TTC

What is claimed is:

1. A DNA comprising an isolated and purified western equine encephalitis (WEE) virus cDNA fragment coding for infectious western equine Encephalitis virus genome.

2. A DNA according to claim 1 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

3. The DNA according to claim 1, wherein said cDNA fragment contains a deletion in the E3-E2 cleavage site.

4. The DNA according to claim 3, wherein said cDNA fragment deletion is 5 amino acids at the E2-E2 cleavage site said amino acids being Arg Arg Pro Lys Arg.

5. The DNA according to claim 4 wherein said cDNA fragment further contains a suppressor mutation.

6. The DNA according to claim 5 wherein said suppressor mutation is a substitution of glutamic acid at codon 182 of E2 to lysine.

7. The DNA according to claim 5 wherein said suppressor mutation is a substitution of glutamic acid at codon 181 of E2 to lysine.

8. The DNA according to claim 2 wherein said promoter is T7 promoter of pBluescript KS and said DNA is pWE2000.

9. An infectious WEE virus RNA transcript encoded by the cDNA fragment of claim 1.

10. A DNA according to claim 5 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

11. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 5.

12. A DNA according to claim 6 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

13. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 6.

14. A DNA according to claim 7 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

15. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 7.

16. Infectious WEE virus particles containing an RNA transcript according to claim 9.

17. Attenuated WEE virus particles containing an RNA transcript according to claim 11.

18. Attenuated WEE virus particles containing an RNA transcript according to claim 13.

19. Attenuated WEE virus particles containing an RNA transcript according to claim 15.

20. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated western equine encephalitis virus according to claim 17.

21. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated Western Equine Encephalitis virus according to claim 18.

22. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated western equine encephalitis virus according to claim 19.

23. A DNA comprising an isolated and purified venezuelan equine encephalitis virus IE variant (VEE IE) cDNA fragment coding for infectious venezuelan equine encephalitis virus IE variant virus genome.

24. A DNA according to claim 23, wherein said cDNA fragment has the sequence of SEQ ID NO:2 or a portion thereof, or an allelic portion thereof.

25. The DNA according to claim 23, wherein said cDNA fragment contains a deletion in the E3-E2 cleavage site.

26. A DNA according to claim 25, wherein said cDNA fragment contains a deletion according to

claim 25 wherein said deletion is four amino acids said amino acids being Arg Gly Lys Arg.

27. A DNA fragment according to claim 26 wherein said VEE IE cDNA further contains a suppressor mutation.

28. A DNA according to claim 23 wherein said VEE IE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

29. An infectious VEE IE virus RNA transcript encoded by the cDNA fragment of claim 23.

30. A DNA according to claim 27 wherein said VEE IE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

31. An attenuated VEE IE virus RNA transcript encoded by the cDNA fragment of claim 27.

32. Infectious VEE IE virus particles containing an RNA transcript according to claim 29.

33. Attenuated VEE IE virus particles containing an RNA transcript according to claim 31.

34. A live attenuated venezuelan equine encephalitis virus IE variant (VEE IE) virus vaccine comprising attenuated venezuelan equine encephalitis virus IE variant virus according to claim 33.

35. A bivalent alphavirus vaccine comprising live attenuated western equine encephalitis (WEE) virus comprising an attenuating mutation and live

attenuated venezuelan equine encephalitis virus IE variant (VEE IE) comprising an attenuating mutation.

36. A bivalent alphavirus vaccine according to claim 35 wherein said live attenuated western equine encephalitis virus is chosen from the group consisting of attenuated WEE comprising a deletion in the E3-E2 cleavage site and a substitution of glutamic acid at codon 182 of E2 to lysine, and attenuated WEE comprising a deletion in the E3-E2 cleavage site and a substitution of glutamic acid at codon 181 of E2 to lysine.

37. An attenuated western equine encephalitis virus wherein said virus comprises an attenuating mutation selected from the group consisting of: a C to T change at nucleotide 7 of the 5′ noncoding region of the WEE genome, a A to G change at nucleotide 13 of the 5′ noncoding region of the WEE genome, a T to A change at nucleotide 25 of the 5′ noncoding region of the WEE genome, and a deletion of an A at nucleotide 22 of the 5′ noncoding region of the WEE genome.

38. A pharmaceutical formulation comprising attenuated WEE virus particles according to claim 17 in an effective immunogenic amount in a pharmaceutically acceptable carrier.

39. A pharmaceutical formulation comprising attenuated WEE virus particles according to claim 18 in an effective immunogenic amount in a pharmaceutically acceptable carrier.

40. A pharmaceutical formulation comprising attenuated WEE virus particles according to claim 19 in an effective immunogenic amount in a pharmaceutically acceptable carrier.

41. A pharmaceutical formulation comprising attenuated VEE IE virus particles according to claim 33 in an effective immunogenic amount in a pharmaceutically acceptable carrier

42. An attenuated chimeric virus comprising nonstructural sequences from a first alphavirus and structural sequences from a second alphavirus resulting in attenuation of the second alphavirus.

43. An attenuated chimeric virus according to claim 42 wherein wherein said first alphavirus is western equine encephalitis virus and said second alphavirus is chosen from the group consisting of: Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Ross River virus, Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus, venezuelan equine encephalitis virus, and Whataroa virus.

44. An attenuated second virus according to claim 43 wherein said second alphavirus is eastern equine encephalitis virus.

45. An attenuated chimeric second virus according to claim 42 wherein said first alphavirus is

Venezuelan equine encephalitis virus variant IE and said second alphavirus is chosen from the group consisting of: venezuelan equine encephalitis virus, western eqine encephalitis virus, eastern equine encephalitis virus, Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Ross River virus, Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virusand Whataroa virus.

46. An attenuated chimeric second virus according to claim 44 wherein said second virus is venezuelan equine encephalitis virus variant IA.

47. An attenuated chimeric second virus according to claim 42 wherein said first alphavirus is venezuelan equine encephalitis virus variant IA and said second alphavirus is chosen from the group consisting of: Venezuelan equine encephalitis virus, western eqine encephalitis virus, eastern equine encephalitis virus, Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Ross River virus, Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus, and Whataroa virus.

48. An attenuated chimeric second virus according to claim 47 wherein said second virus is venezuelan equine encephalitis virus variant IE.

49. An attenuated chimeric second virus according to claim 47 wherein said second virus is Venezuelan Equine Encephalitis virus variant IIIA.

50. An attenuated virus vaccine comprising chimeric virus according to claim 42 wherein said vaccine is directed against said second alphavirus.

51. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 17 wherein said attenuated WEE is inactivated.

52. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 18 wherein said attenuated WEE is inactivated.

53. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 19 wherein said attenuated WEE is further inactivated.

54. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 33 wherein said attenuated VEE IE is further inactivated.

55. An inactivated eastern equine encephalitis virus (EEE) vaccine comprising attenuated EEE according to claim 44 wherein said attenuated virus is further inactivated.

56. An inactivated venezuelan equine encephalitis virus variant IA (VEE/IA) vaccine comprising attenuated VEE/IA according to claim 46 wherein said attenuated virus is further inactivated.

57. An inactivated venezuelan equine encephalitis virus variant IIIA (VEE/IA) vaccine comprising attenuated VEE IIIA according to claim 49 wherein said attenuated virus is further inactivated.

58. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 48 wherein said attenuated virus is further inactivated.

59. A method for expressing a protein said method comprising cloning a gene encoding said protein into a an attenuated virus replicon, said replicon chosen from the group consisting essentially of attenuated WEE and attenuated VEE IE wherein transcription of said replicon yields RNA capable of infecting a cell in which said protein is to be expressed.

60. A method for the diagnosis of western equine encephalitis virus (WEE) infection comprising the steps of :

(i) contacting a sample from an individual suspected of having a WEE infection with all or a unique portion of WEE; and

(ii) detecting the presence or absence of a WEE infection by detecting the presence or absence of a complex formed between WEE and antibodies specific therefor in the sample.

61. A method for the diagnosis of venezuelan equine encephalitis virus variant IE (VEE IE) infection comprising the steps of :

(i) contacting a sample from an individual suspected of having a VEE IE infection with all or a unique portion of VEE IE; and

(ii) detecting the presence or absence of a VEE IE infection by detecting the presence or absence of a complex formed between VEE IE and antibodies specific therefor in the sample.

62. A method for the diagnosis of western equine encephalitis virus (WEE) from a sample using the polymerase chain reaction, said method comprising:

(i) extracting RNA from the sample;

(ii) reverse transcribing the RNA of (i) to DNA;

(iii) contacting said DNA with

(a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to WEE DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said DNA;

(v) contacting said first DNA product with a reaction mixture comprising:

(a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) fractionating said first and second DNA products generated from said WEE DNA; and

(ix) detecting said fractionated products for the presence or absence of WEE in a sample.

63. A method for the diagnosis of venezuelan equine encephalitis variant IE virus (VEE IE) from a sample using the polymerase chain reaction, said method comprising:

(i) extracting RNA from the sample;

(ii) reverse transcribing said RNA of (i) to DNA;

(iii) contacting said DNA with

(a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to VEE IE DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said DNA;

(v) contacting said first DNA product with a reaction mixture comprising:

(a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) fractionating said first and second DNA products generated from said VEE IE DNA; and

(ix) detecting said fractionated products for the presence or absence of VEE IE in a sample.

64. A method for providing protective immunity against a second alphavirus to individuals with pre-existing immunity to a first alphavirus said method comprising administering to said individuals an effective amount of live attenuated second alphavirus.

65. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated western equine encephalitis virus.

66. The method of claim 65 wherein said western equine encephalitis (WEE) virus is chosen from the group consisting of: attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a C to T change at nucleotide 7 of the 5′ noncoding region of the WEE genome, attenuated WEE having a A to G change at nucleotide 13 of the 5′ noncoding region of the WEE genome, attenuated WEE having a T to A change at nucleotide 25 of the 5′ noncoding region of the WEE genome, and attenuated WEE having a deletion of an A at nucleotide 22 of the 5′ noncoding region of the WEE genome.

67. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated venezuelan equine encephalitis virus variant IE.

68. A WEE infection diagnostic kit comprising primers specific for WEE and ancillary reagents suitable for use in detecting the presence or absence of WEE in a mammalian sample.

69. A VEE IE infection diagnostic kit comprising primers specific for VEE IE and ancillary reagents suitable for use in detecting the presence or absence of VEE IE in a mammalian sample.

70. An isolated and purified Venezuelan equine encephalitis variant IIIA cDNA encoding the VEE IIIA structural genes.

71. An amino acid fragment encoded by the cDNA fragment according to claim 70.

72. A polypeptide encoded by the amino acid fragment according to claim 71.

Asssembly ꞉m. ule WE3'-17 1 / 7

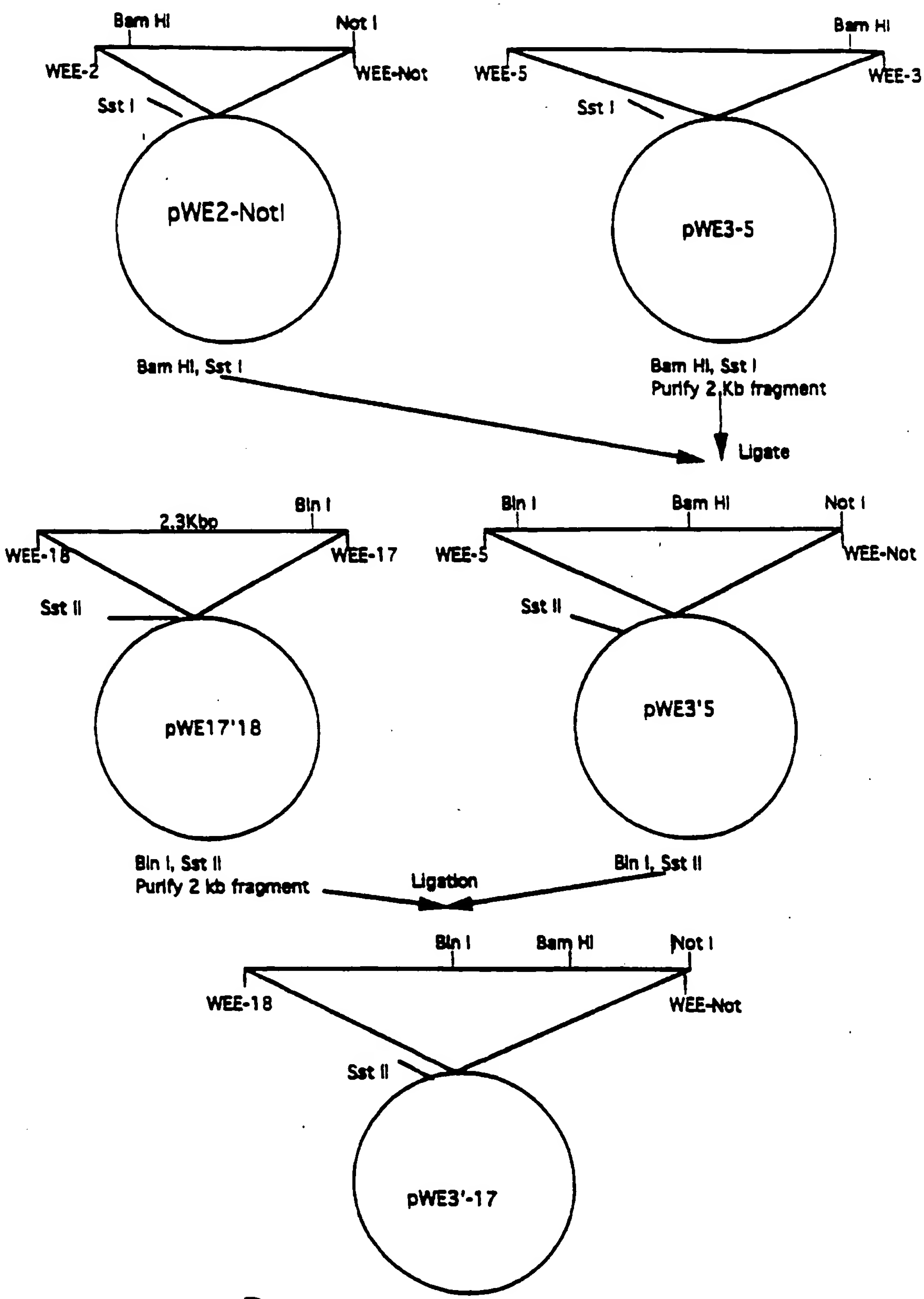

FIGURE 1A

Assembly c noc ɹ pWE5'-18 2 / 7

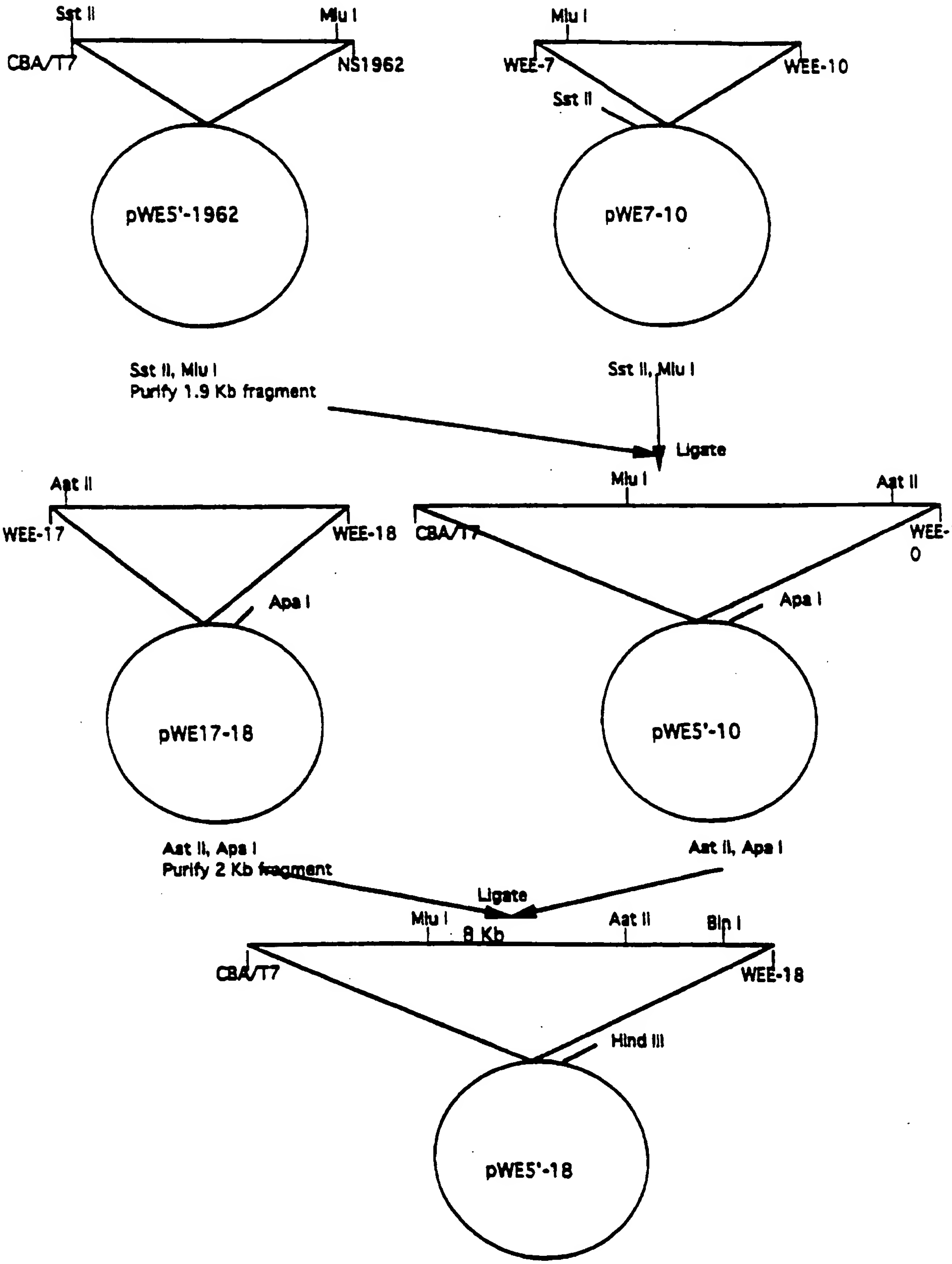

FIGURE 1B

Assembly of pWE2000 full length cDNA clone of western equine encephalitis virus from modules pWE5'-18 and pWE3'-17

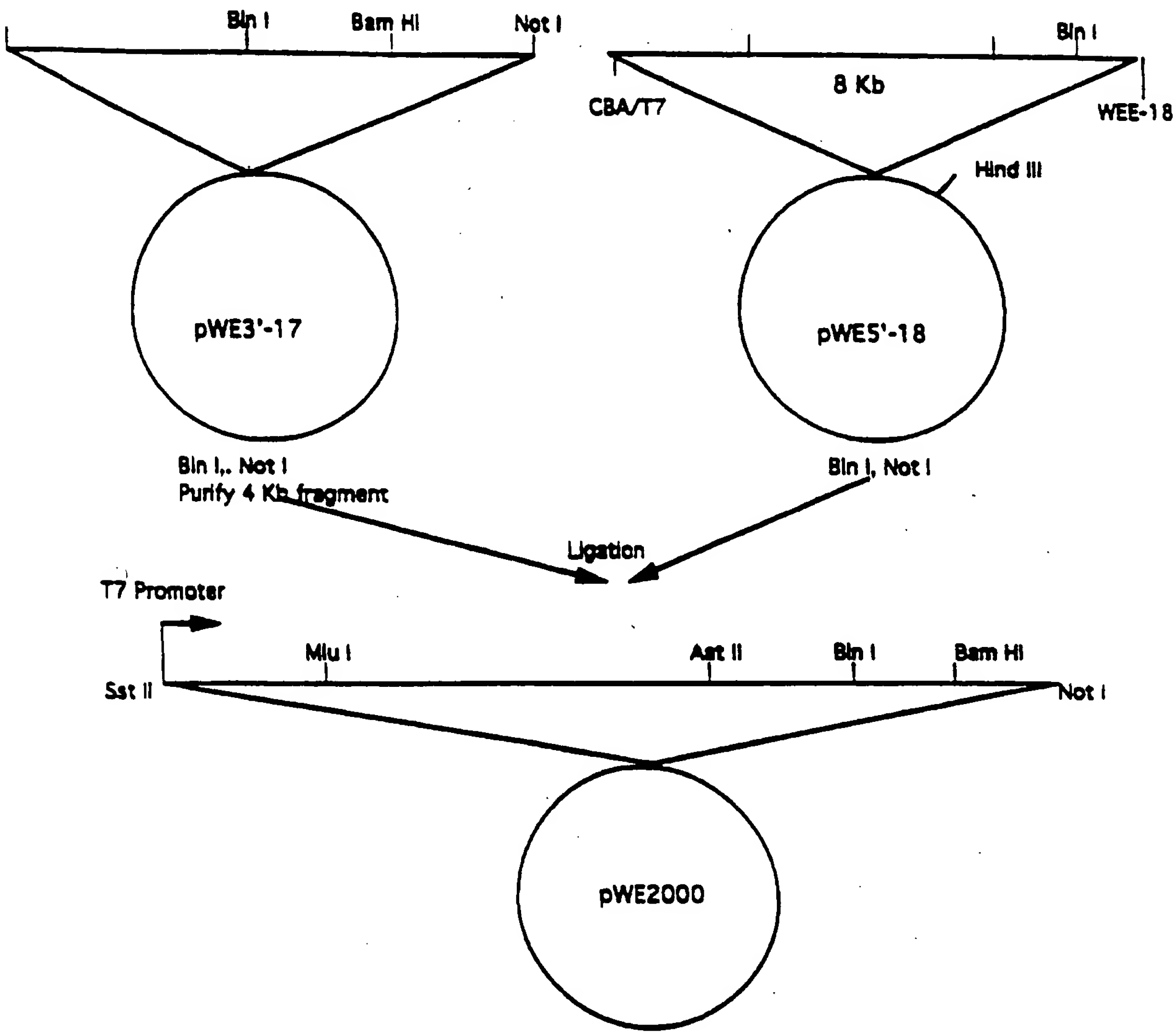

FIGURE 1C

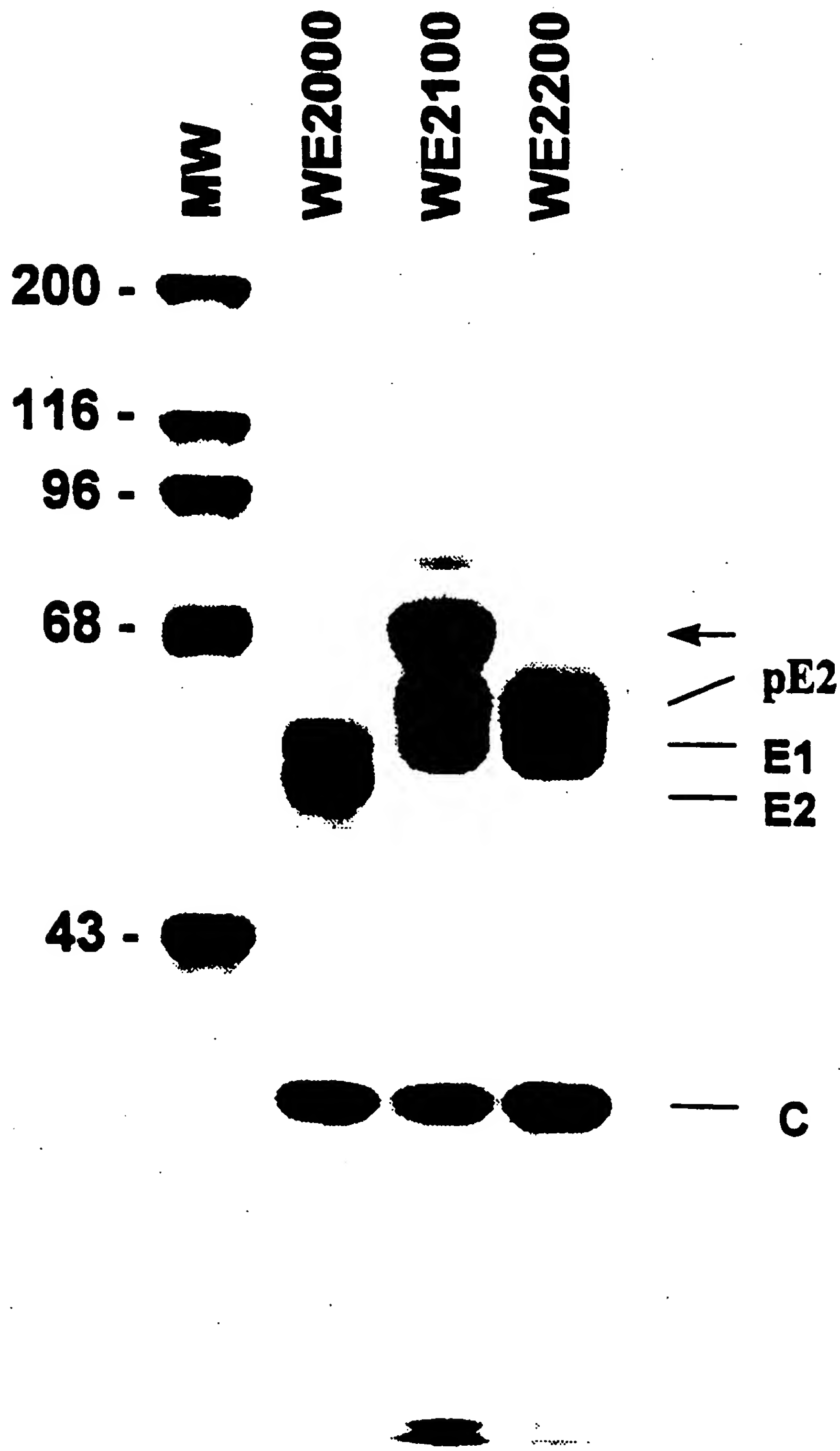

FIGURE 2

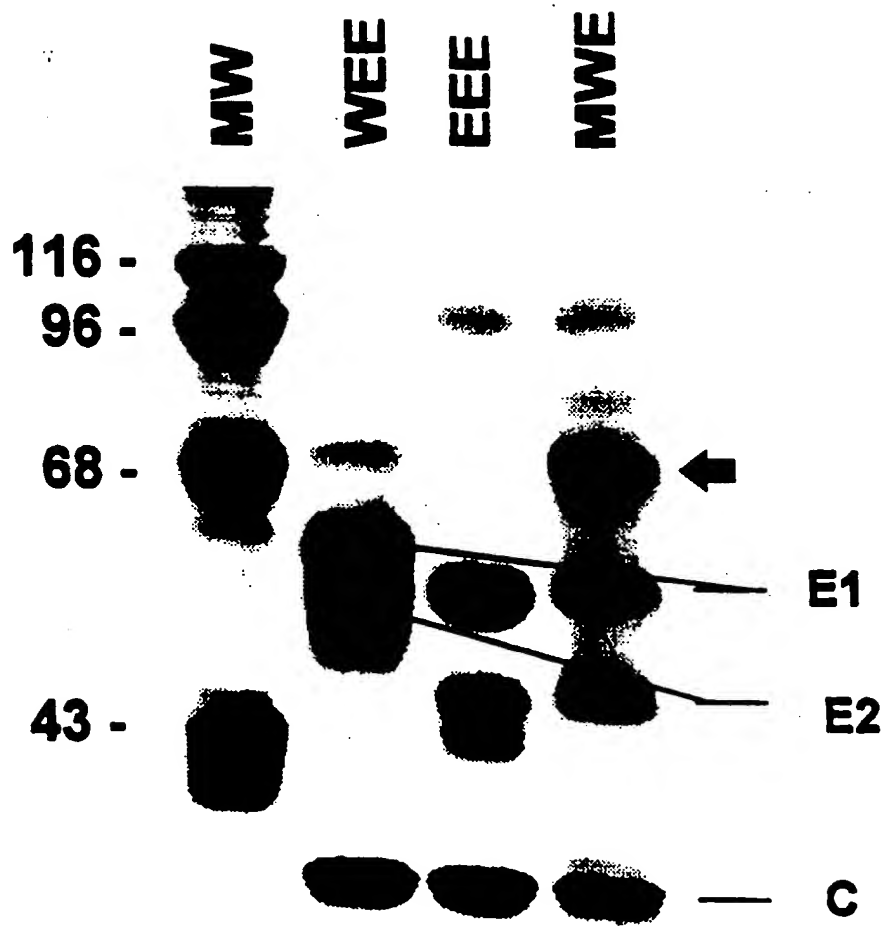

FIGURE 3

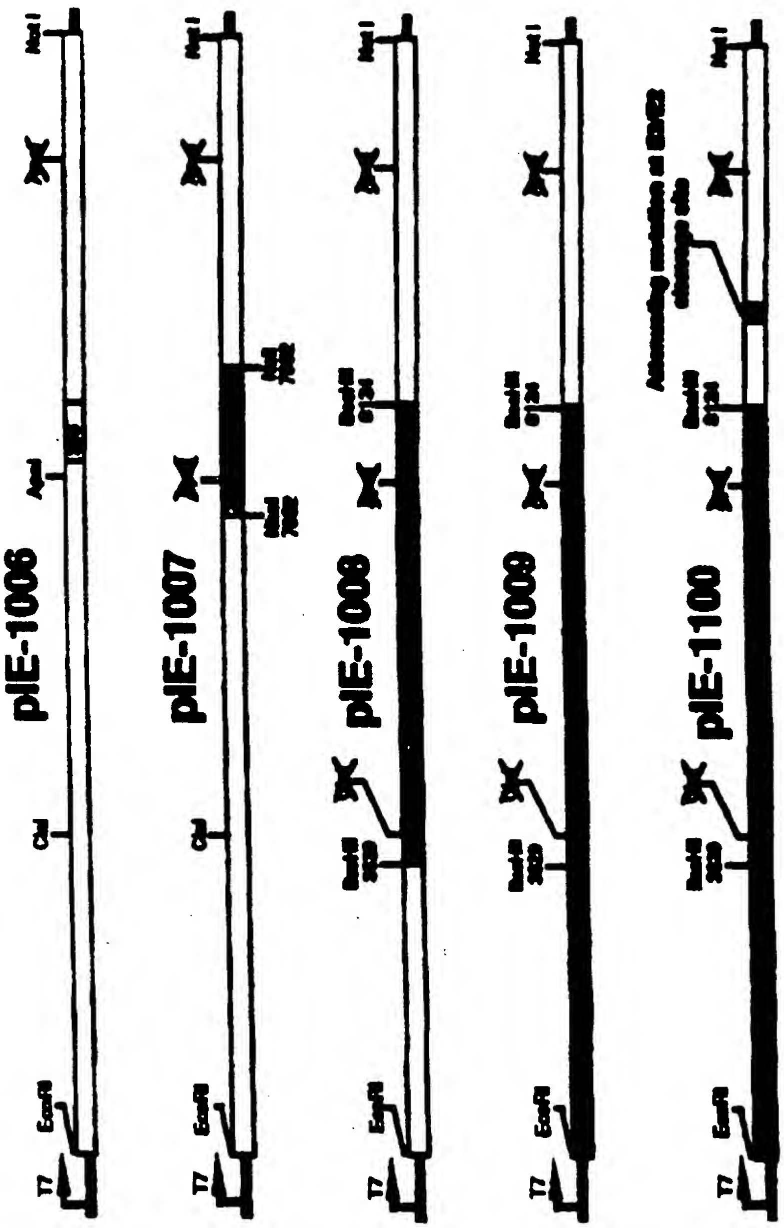

Figure 4

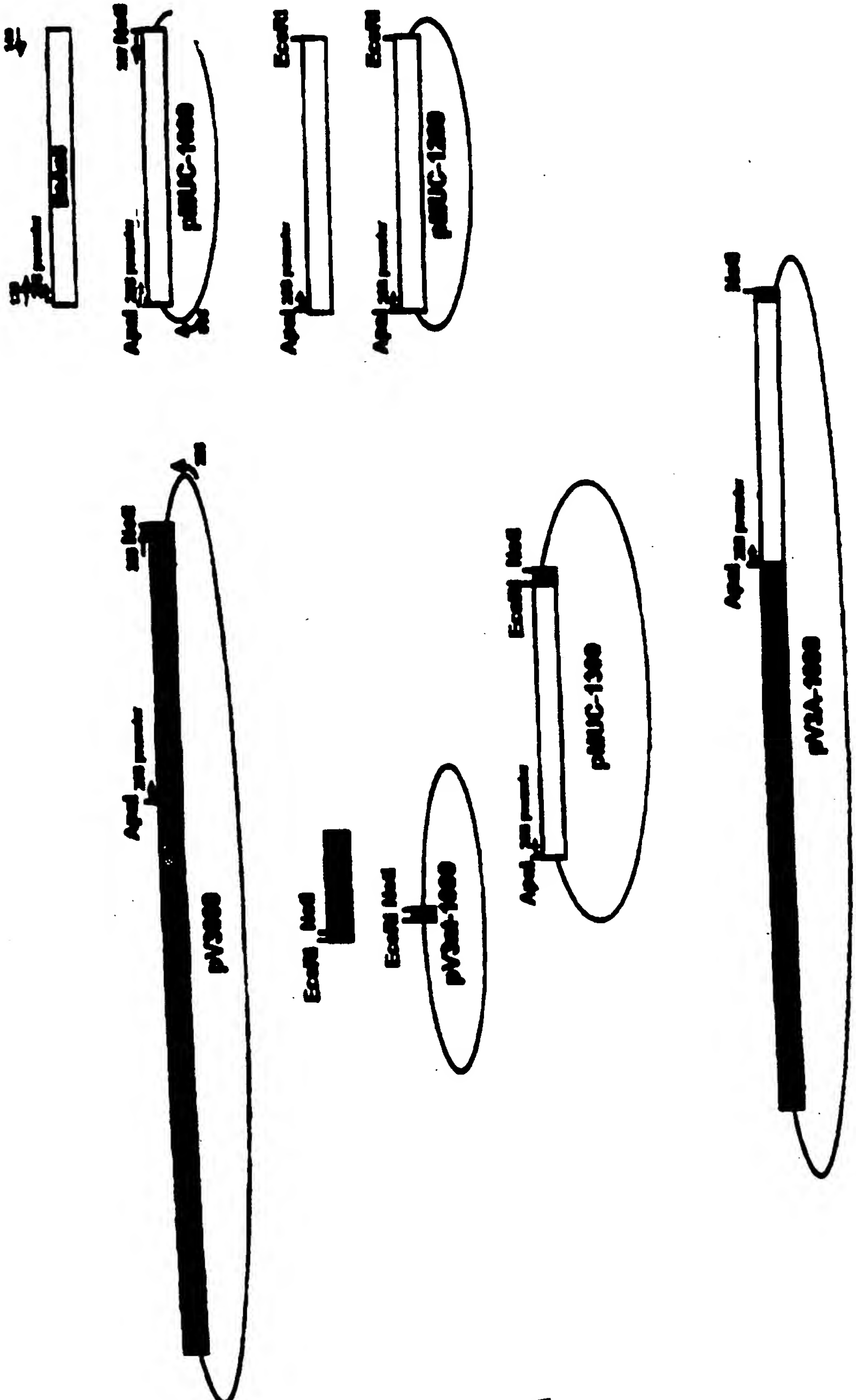

FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10645

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/40, 7/01, 7/04, 15/86; A61K 39/193.
US CL :536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Aps, Biosis, Cab, Derwent WPI. Search terms: western, equine(w)enceph?, attenuat?, venezuelan, genom?, rna, clon?, sequenc?, dna, dnas, "E2", "E3", wee.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAIZER, G. et al. Isolation and preliminary characterization of mutants of Western equine encephalomyelitis virus with altered virulence in chickens. Biological Abstracts. 1985. Vol. 80, No. 5, page AB-506, Abstract no. 41179, see entire abstract.	16
Y	US 5,185,440 A (DAVIS et al) 09 February 1993, see entire document, particularly column 7, line 66 through column 8, line 1.	1-3, 8, 9, 16, 59
Y	US 5,505,947 A (JOHNSTON et al) 09 April 1996, see particularly column 3, lines 50-54, Abstract, and Examples 1 and 2.	3, 59

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 1998	06 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Deborah Freese for MARY E. MOSHER, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEAVER, S.C. et al. A comparison of the Nucleotide Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related RNA Viruses. Virology. 1993. Vol. 197, pages 375-390. See figures 3 and 4.	1-3, 8, 9, 16, 59
Y	HAHN, C.S. et al. Western equine encephalitis virus is a recombinant virus. Proceedings of the National Academy of Sciences USA. August 1988. Vol. 85, p. 5997-6001. See Figure 1.	1-3, 8, 9, 16, 59
A	STRAUSS, J.H. et al. The Alphaviruses: Gene Expression, Replication, and Evolution. Microbiological Reviews. September 1994. Vol. 58, No. 3, pages 491-562.	1-22, 37-40, 51-53, 59

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 37-40, 51-53, 59

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group 1, claims 1-22, 37-40, 51-53, 59, drawn to a first product DNA encoding an infectious genome of Western Equine Encephalitis (WEE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 2, claims 23-34, 41, 54, 59 drawn to second product DNA encoding an infectious genome of Venezuelan Equine Encephalitis IE variant (VEE IE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 3, claims 35-36, drawn to third product, combination vaccine comprising attenuated WEE and attenuated VEE IE

Group 4, claims 42-50, 55-58, drawn to fourth product, chimeric alphavirus and vaccine.

Group 5, claims 62, 68, drawn to fifth product, WEE specific primer kit, and method of use.

Group 6, claims 63, 69, drawn to sixth product, VEE IE specific primer kit, and method of use

Group 7, claims 70-72 , drawn to seventh product, DNA encoding structural genes of VEE variant IIIA and products encoded.

Group 8, claims 60 , drawn to method of diagnosing WEE using antigen.

Group 9, claims 61, drawn to method of diagnosing VEE IE using antigen.

Group 10, claims 64-67, drawn to method of vaccinating immune individual using a second alphavirus.

The inventions listed as Groups 1-10 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

In groups 1 and 2, the special technical feature in each case is the DNA encoding an infectious genome of a particular virus; since groups 1 and 2 pertain to two distinct viruses, they involve different special technical features.

Group 3 does not have the corresponding special technical feature, because the attenuated viruses required in group 3 are not limited to those made by using the DNAs of groups 1 or 2; for example claim 35 encompasses combination vaccines using viruses attenuated by propagation in culture.

Group 4 does not have the corresponding special technical feature, because it is not limited to either of the viruses encoded by the DNAs of group 1 or group 2; for example claim 42 encompasses a chimera made from Sindbis and Semliki Forest viruses.

Groups 5 and 6 do not have the corresponding special technical feature, because they require small, specific primers, not infectious full-length genomic materials.

Group 7 does not have the corresponding special technical feature, because is drawn to a sub-genomic fragment of a third virus.

Groups 8 and 9 do not have the corresponding special technical feature, because they are not limited to materials of groups 1 or 2; for example claim 60 encompasses a method using viral antigens produced by propagation of native WEE in standard tissue culture.

Group 10 does not have the corresponding special technical feature because it is not limited to the viruses made using the DNAs of groups 1 or 2; for example claim 64 encompasses a method of vaccinating a Sindbis-immune monkey

against Semliki Forest virus.